SPECIFICATION OF SPINAL NEURON CONNECTIONS IN *XENOPUS LAEVIS*

THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

by

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DECLARATION

I declare that this thesis contains no material which has been submitted or accepted for the award of any other degree or diploma in any university. The original work described in Chapters 2, 3 and 4 is mine alone, that in Chapter 5 was done in collaboration with others who are named in that Chapter.

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ABSTRACT

1. The hindlimb bud of *Xenopus laevis* tadpoles was replaced at stage 49, prior to motor axon invasion and the naturally occurring period of motoneuron death, by the forelimb bud from a stage 50 donor. About 10% of the transplanted buds underwent excellent morphological and anatomical differentiation, giving rise to a forelimb, complete to at least the level of the shoulder, in the hindlimb position. Developmental abnormalities arising from the operation included hindlimb regeneration, duplication of the distal forelimb, and both regeneration and duplication.

2. All well-formed forelimbs received innervation from the complement of 3 lumbar nerves that normally supply the hindlimb, however these nerves were of reduced diameter in comparison with those supplying the contralateral hindlimb. The 3 lumbar nerves generally, but not always, formed a single trunk prior to entering the transplanted forelimb. The entry point and branching pattern of the main mixed and cutaneous nerves was typical of a normal left or right forelimb, from the level of the shoulder to the finger-tips.

3. Motoneurons on the operated side were identified morphologically, and by retrograde transport of horseradish peroxidase injected to the forelimb after metamorphosis. The lateral motor column (LMC) on the operated side was of normal length, and contained on average 46% as many motoneurons as on the unoperated side (range 39 - 56%, N = 10).

4. Dorsal root ganglia 8, 9 and 10 contained on average 87% as many neurons on the operated, as opposed to the unoperated side (range 79 - 104%, N = 6). It was concluded that lumbar motor and dorsal root ganglion neurons survived the naturally occurring period of death in reduced numbers, following innervation of a forelimb from the beginning. The reduction in neuron numbers was not proportional to the reduction in mass of the peripheral field.

5. In some tadpoles the hindlimb bud was replaced at stage 49 with several myotomes. The myotomes became atrophic and disappeared prior to stage 58. The number of motoneurons in the LMC on the operated side after metamorphosis did not exceed that surviving unilateral limb amputation in control animals.

6. Whereas forelimbs in the hindlimb position were almost normal in size, hindlimbs transplanted in place of a forelimb appeared atrophic.

7. The nuclear areas of 1791 motoneurons at the level of the nucleolus were measured in a total of 6 spinal cords providing innervation to a transplanted forelimb. The size distribution of nuclear areas was positively skewed and leptokurtic in all 12 LMCs. The distributions were significantly different for motoneurons on the operated, as opposed to the unoperated side, in 2 of the 6 spinal cords (Kolmogorov-Smirnov test, 5% level). It was concluded that many lumbar motoneurons had attained a normal level of maturity, in terms of nuclear size, after innervating a forelimb from the onset.

8. All transplanted forelimbs moved in co-ordination with the swimming movements of the hosts' hindlimb, regardless of their orientation. Identification of lumbar motoneurons projecting to 3 specific regions of the forelimb provided some evidence of selective innervation. These observations therefore did not furnish evidence of a peripheral influence on the acquisition of central connections by limb motoneurons.

9. The reflex withdrawal of the normal forelimb was not accompanied by withdrawal of the transplanted forelimb. Mechanical stimuli delivered to the skin of the transplanted forelimb elicited a small flexion response in the limb, unaccompanied by withdrawal reflexes in other limbs, as judged behaviourally. Swimming could be elicited by a strong stimulus delivered to any part of the skin. Wiping reflexes could be elicited in *Xenopus* by noxious chemicals delivered to the skin. Chemical stimuli applied to the area of skin normally wiped by the hindlimb, elicited movements in transplanted forelimbs. These observations suggest that reflex connections may be made between the skin of the trunk and lumbar motoneurons supplying a transplanted forelimb, but not between the skin of a transplanted forelimb and the lumbar motoneurons, nor the brachial motoneurons when the forelimb is in the hindlimb position.

10. When deprived of serum, cells of the rat pheochromocytoma line PC 12 die, unless provided with the chemical nerve growth factor (NGF). The initial rate of uptake of $^{24}Na^{+}$ in response to NGF was shown to increase almost 2-fold over a period of 90 minutes after NGF presentation to serum-deprived PC 12 cells. This may be the basis of the known effect of NGF in stimulating the Na^{+}-K^{+} pump in the cell line. It was shown that in addition to NGF, serum also stimulated the Na^{+}-K^{+} pump activity, and the stimulation could be reduced by the diuretic amiloride. It was concluded that some of the ionic events initiated by NGF in this cell line were common to other growth factors.

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CHAPTER ONE

INTRODUCTION : SPECIFICATION OF SPINAL NEURON CONNECTIONS DURING DEVELOPMENT

1.1 DEVELOPMENT AND INHERITANCE : HISTORICAL CONTEXT

Biological development is the sequence of events by which the zygote reaches maturity. From the preformation viewpoint, development consisted of the unfolding of structures already present in the embryo, a problem simply of growth. In opposition to the pre formation theory, the 'epigenetic' view regarded development as the appearance of structures which did not previously exist in the embryo (see Waddington, 1956, 1969). The extreme preformation view forced the conclusion that the embryo contained not one, but all succeeding generations in miniature. The notion of a preformed individual in the sperm or egg was eventually disproven by direct observation, during the seventeenth century.

In 1868 Darwin published his theory of pangenesis, in an attempt to account for the existing evidence concerning variation and inheritance. He suggested that "the whole organization, in the sense of every separate atom or unit, reproduced itself". Although it was appreciated that reproduction entailed more than the parent simply acting as a form of template for the offspring, the events had not been fully established in terms of the cell theory at that time. It was therefore proposed that the evident point to point correspondence between parent and offspring was effected by 'gemmules' released from every part of the body, and transmitted via the bloodstream to the germ cells. Any form of direct continuity between parent and offspring was,

however, later disproven experimentally by Driesch (1891). Driesch separated the blastomeres of sea urchin embryos at the 2 cell stage, and each cell gave rise to a complete individual.

The theory of pangenesis also could not adequately account for reproduction where the offspring inherited characters not expressed in either parent. Mendel showed in those cases that inheritance was from both parents but without blending of the inherited factors. Mendel's Law of Segregation was later coupled to the assertion of Weisman (1904), that the organism could be divided into the germ plasm, containing the inherited factors, and the soma or body, whose form was dictated by the germ plasm. This placed strong emphasis on the inherited factors in the cell nucleus, and in 1953 the model of DNA structure proposed by Watson and Crick showed that segregation of these factors could be explained in terms of the unique mode of separation of the nuclear structures at cell division.

In light of the success in elucidating the basis of nuclear inheritance in terms of DNA structure, development has commonly been regarded as a problem of translation of the inherited genetic code, or 'information' transmission by genes (Apter, 1966; Williams, 1966; Dawkins, 1976). This might be correct if Weismann's division of the organism into the germ and soma plasm was valid. There is, however, substantial evidence to suggest that changes in structural organization at the cortical and supracellular levels are themselves self-propagating, independent of any changes in the cell nucleus (Sonneborn, 1930, 1963, 1967, 1970). In fact the cell nucleus can only replicate itself (Sonneborn, 1951).

Although changes in the cell cortex must be compatible with the cell nucleus and vice-versa, one does not necessarily direct the other's development entirely.

Although comparatively well understood, replication of nuclear material is but one step in the dynamic chain of events at the nuclear, cellular and supracellular levels leading to reproduction of a metazoan organism. As an 'open' physiochemical system, interactions between the inherited structures and the external environment give rise to successive increases of structural order. The inheritance sets the limits within which these interactions with the surroundings may occur (Waddington, 1954). There is then no reason to suppose the existence of an inherited program governing the step-by-step development of the organism (Goodwin, 1977).

It is implicit in epigenetic development that the organism undergoes a net increase in structural order, or stability, yet this is highly improbable under equilibrium conditions. If, however, there is unequal exchange of matter and energy with the surroundings, increases in structural order become likely. The importance of non-equilibrium conditions as a 'source' of organization is now widely recognized in physiochemical systems (Nicolis and Prigogine, 1977).

Every living cell must exchange materials with its surroundings in order to survive, grow and reproduce. During metazoan development, where the surroundings will include other cells, there may ensue a competition for resources, forcing each cell along common or divergent developmental paths, and coincidentally fitting each to its place in the adult form. The main theme of this introduction will be the role of

competition between neurons, leading to the formation of an adult nervous system, without the existence of a rigidly predetermined plan of development.

1.2 DEVELOPMENTAL STUDIES OF THE NERVOUS SYSTEM

Early workers revealed that the nervous system, in common with most other tissues of the body, was composed of separate cells (Harrison, 1910; Ramón y Cajal, 1928) with distinctive forms and precise interconnections. Subsequent work has confirmed and extended these observations, particularly in the invertebrates. For example, the morphology and synaptic connections of almost all of the 300 neurons of the nervous system of the small nematode *C. elegans* have been elucidated (Ward, Thomson, White and Brenner, 1975; Ware, Clarke, Crossland and Russel, 1975; White, Southgate, Thomson and Brenner, 1976).

It has been difficult, even in principle, to reconcile the evident precision of connections at the level of the single neuron with the functional adaptability of the nervous system as a whole, and it has been suggested that nervous function may be more dependent on diffuse patterns of impulse activity, or field potentials, rather than on stable, organized connections between neurons (Lashley, 1929; John, 1972). Although the patterning of nerve impulses may be of some importance (Sinclair, 1955; Melzack and Wall, 1962; Székely, 1974), it is generally accepted that specificity of connections is the principal substrate for proper nervous function (Sperry, 1951, 1965), and as a corollary, a better understanding of the principles

of development of these connections will increase our knowledge of nervous function.

The development of neuromuscular, somatosensory and retinotectal systems has been frequently reviewed (Hughes, 1968; Gaze, 1970; Grinnell, 1977; Jacobson, 1978) not only as regards normal development, but also during reinnervation, which is often used as a model of development. Since the experiments reported in Chapters 2, 3 and 4 concern the developing spinal cord, the emphasis here is on the neuromuscular (reviewed by Purves, 1976; Hollyday, 1980 a; Landmesser, 1980; Lømo, 1980; Mark, 1980; Oppenheim, 1981; Grinnell and Herrera, 1981) and somatosensory (reviewed by Baker, 1978) systems. Some reference is also made to relevant examples from the retinotectal system (reviewed by Gaze, 1978).

1.3 MORPHOGENESIS OF THE SPINAL CORD

The cells destined to form the nervous system are committed to that fate by the end of the morphogenetic movements of gastrulation. During gastrulation the spherical blastula invaginates, giving rise to the ectodermal and endodermal cell layers. An approximately oval-shaped region of ectoderm separates from the surrounding presumptive epidermis, to form the neural plate. The neural plate then sinks below, and is covered by surrounding ectoderm, and then rolls up to form the neural tube. This is accompanied by the segmentation of the surrounding mesoderm, and migration of cells from the margins, to form the dorsal root ganglia (Harrison, 1904).

The motoneurons arise by detachment from the ependymal layer of the basal place (Wenger, 1950). The neurons migrate laterally, to aggregate in the region defined as the motor column. During this migration they can be retrogradely labelled with horseradish peroxidase as their axons pass from the ventral roots and grow towards the limb bud (Farel and Bemelmans, 1980). The medial somatic motor column extends through all cord segments and contains motoneurons which supply the axial, or trunk muscles. The lateral somatic motor column (LMC) is confined to segments adjacent to the limb, or fin, and contains motoneurons which supply the appendicular musculature. In *Gallus* brachial cord the segments in which they are located correspond to those from which the limb mesoderm is derived (see Chevallier, Kieny and Mauger, 1977, 1978; Chevallier, 1979).

The medial motoneurons can form transmitting junctions with the muscles of a transplanted limb (Morris, 1978) but fail to provide them with trophic long term support. Muscles in limbs transplanted to thoracic regions in amphibia (Harrison, 1907; Detwiler, 1920a; Miner, 1956; Hollyday and Mendell, 1975) and chickens (Székely and Szentagothai, 1962; Morris, 1978) show fibrillations and become atrophic.

In both the amphibian and avian LMC there are clear rostrocaudal and mediolateral gradients of production. The first motoneurons settle medially and later arrivals migrate between the first assembled cells, to settle more laterally (Prestige, 1973; Hollyday and Hamburger, 1977).

In fish the primary motor system persists into adult life with the addition of motoneurons as the animal grows (Leonard, Coggeshall and Willis, 1978; Birse, Leonard and Coggeshall, 1980).

The neural crest in the embryo gives rise to the dorsal root ganglia, Schwann cells (Harrison, 1904), autonomic ganglia, and other derivatives (reviewed by Weston, 1970). This takes place by migration of neural crest cells to their final sites, with subsequent cell proliferation. A dorsolateral stream beneath the epidermal ectoderm gives rise to a uniform, unsegmented aggregation of cells within the ectodermal layer. A ventral stream migrates into the spaces between the somites to form the DRG. Removal or addition of somites results in corresponding changes in the number of DRG (Detwiler, 1934). When first recognizable in the amphibian *Xenopus* at about stage 39, each DRG consists of 6-10 neuroblasts (Hughes and Tschumi, 1958). Cell proliferation subsequently enlarges the population to the adult total of about 10,000 cells in the lumbar ganglia, by the time of metamorphosis (Prestige, 1965).

In amphibians the development of the DRG is preceded by the formation of a primary sensory system, consisting of the Rohon-Beard cells (Hughes, 1957). They form almost continuous double medio-dorsal rows along the surface of the spinal cord. Peripherally they innervate the skin with 'free' endings, and centrally they give rise to ascending and descending axons in the dorsal tract (Roberts and Clarke, 1982). The cells are lost prior to the completion of development of the DRG (Hughes, 1957).

The lateral motor column (LMC) in chicken brachial cord is generated between days 2.5 to 4 of development, as shown by thymidine dating (Hollyday and Hamburger, 1977). In mouse cervical cord, the motoneurons are generated between days 818 and 11.5, with 90% being produced between days 9 and 10.5 (Sims and Vaughn, 1979). They are the first neurons to be generated in the cord. In the amphibian the lumbar LMC is generated between stages 49 and 52, a period of about 10 days (Prestige, 1973).

In chicken spinal cord at the 4th day of development the motoneurons form a single column of uniform width throughout the spinal cord segments. Beginning on day 5 the medial and lateral somatic motor columns arise by segregation of neurons from the single column, and the neurons within each begin to differentiate (Levi-Montalcini, 1950, 1964).

Similarly, a continuous uniform motor column is evident early in foetal mammalian development. In rats the segregation of motoneurons into separate columns is virtually completed by the 17th day (Angulo y Gonzalez, 1940) and in the mouse by the 14th day (Harris, 1965).

In larval amphibia these events are preceded by the formation of a primary motor system (Hughes, 1959). As the first motoneurons are generated they form connections in rostrocaudal order with successive myotomes (Blackshaw and Warner, 1976a). This matches the sequence in which the myotomes arise by segmentation of the

presumptive mesoderm, which continues to late swimming stages (Hamilton, 1969; Pearson and Elsdale, 1979). At later stages large numbers of newly generated motoneurons migrate from the ventricular region, to aggregate within the lateral motor column (Prestige, 1973). Although continuous for the length of the spinal cord (Székely, 1976) the lateral motor column (LMC) is much larger at brachial and lumbar levels, where the motoneurons supply the developing limbs. In urodeles this enlargement is inconspicuous, or absent (Székely and Czéh, 1967). At other segmental levels the developing secondary musculature probably receives innervation from the increasing number of motoneurons evident at these levels (Nieuwkoop and Faber, 1967). The primary motoneurons innervating the tail degenerate upon its resorbtion towards metamorphosis (Hughes, 1968). The fate of the remainder has not been investigated. The sensory Rohon-Beard cells also degenerate approaching metamorphosis (Hughes, 1957).

1.4 REGIONAL DETERMINATION OF THE SPINAL CORD

Cell proliferation is evident in the neural plate and tube, however the total volume of the embryo changes only slightly. The proliferating cells are attached at all times to a basement membrane, forming the subventricular zone in the neural tube (Sauer, 1935a, b; 1936). Presumptive fate maps obtained by marking cells with vital stains or fine carbon particles (Nieuwkoop, 1955), or more recently by horseradish peroxidase injections to individual blastomeres (Jacobson and Hirose, 1978, 1981; Hirose and Jacobson, 1979), showed that cells tended to remain close to the region where they were generated, and consequently also to their ancestors in the neural plate.

By removal, rotation or translocation of cells in the embryo it has been possible to show whether the cells were embryologically determined for a particular developmental fate at the time of the operation. Large regions of the early neural plate of *Amblystoma* that were rotated about the rostrocaudal axis maintained their original polarity (Roach, 1945; Jacobson, 1964). In the presumptive medulla this led to a reversal of the motor nuclei, nerve roots and major fibre tracts (Jacobson, 1964). At later stages, in the neural tube, the inversion of several lumbosacral segments along the rostrocaudal axis (Lance Jones and Landmesser, 1980, 1981 b) led to a reversal of the normal motor axon paths followed between the cord and limb, so that the motoneurons projected to their usual muscles. Similarly, transplantation of brachial or lumbosacral cord segments to the thoracic level of the neural tube led to limb movements in accordance with the grafts' origins (Detwiler, 1923; Székely, 1963; Straznicky, 1963; Narayanan and Hamburger, 1971). For example, birds with brachial segments in place of the lumbar segments moved their legs in synchrony, in the manner of wing flapping (Straznicky, 1963). Brachial segments exchanged with thoracic segments in the early neural plate of urodeles, however, could mediate normal forelimb movements (Detwiler, 1923; Straznicky and Székely, 1967).

These experiments have shown that at least several motor regions of the nervous system were determined in morphological or functional terms during neural plate or neural tube stages, prior to axon outgrowth. The regions contain the earliest formed neurons of the nervous system (Lamborghini, 1980), which suggests that the critical

events may have occurred at about the time of the final cell division. This is further supported by experiments on determination of the paired Mauthner neurons in the medulla of larvel amphibia. Removal of a large part of the presumptive medulla in the frog at the early gastrula stage did not prevent Mauthner neurons from forming. They only formed after removal of small pieces of presumptive medulla in the late gastrula, and failed to form in the post-gastrula embryo. If the tissue was explanted, Mauthner neurons occasionally formed in both the explant and the cells adjacent to the excised area in the embryo (Stefanelli, 1951).

Determination of neurons in the sensory ganglia has also been experimentally investigated by means of tissue transplantation. Weiss (1942) transplanted an eye to the region of the nose or ear in the urodele amphibian *Triturus*, at mid-larval stages. After metamorphosis a cutaneous stimulus to the transplant eye could elicit a blink reflex in the normal eye, a response normally mediated by the ophthalmic division of the trigeminal nerve. Weiss suggested that the transplant eye had appropriately modified the central relations of the sensory neurons that had by chance reinnervated it. Székely (1959 b) transplanted a limb to the head of *Pleurodeles* larvae, just prior to metamorphosis. Stimulation of the limb failed to produce a corneal reflex, but when its tip was amputated, stimulation of the regeneration blastema evoked a blink response. It was therefore suggested that corneal reflexes evoked from transplant eyes might represent a non-specific response to stimulation of a wound surface.

Székely (1959 a) observed that whereas the trigeminal nerve mediated corneal reflexes in *Triturus*, gill withdrawal reflexes were mediated by the vagus nerve. Experimental animals with 2 vagal ganglia and no trigeminal ganglion responded to corneal stimulation with withdrawal of the gills prior to metamorphosis, and gave corneal reflexes after metamorphosis. Animals with 2 trigeminal ganglia and no vagal ganglion produced normal gill withdrawal reflexes prior to metamorphosis, and no corneal reflexes upon stimulation of the gill area after metamorphosis. It was suggested that some functional specificity existed in the ganglia prior to their transplantation.

A limb bud transplanted to thoracic regions in amphibia (Miner, 1956; Hollyday and Mendell, 1975) or chickens (Székely and Szentágothai, 1962) self-differentiates and receives motor and sensory innervation from only thoracic cord segments. The limb muscles atrophy, but the sensory innervation is maintained.

Cutaneous stimulation of the transplant limb may evoke a withdrawal reflex in the normal ipsilateral limb (Miner, 1956; Székely and Szentágothai, 1962; Hollyday and Mendell, 1975). DRG neurons do not branch to supply both limbs (Hollyday and Mendell, 1975). Miner (1956) reported appropriate withdrawal reflexes evoked in a forelimb by stimulation of a rostrally transplanted hindlimb (see also Székely and Szentágothai, 1962), suggesting that withdrawal is an unspecific response. In contrast Hollyday and Mendell (1975) could not elicit the normal hindlimb extension reflex by stimulation of the homologous area of a transplant forelimb. They suggest that stimulation of only the

distal limb extremities is not discriminatory as it causes flexion in either a forelimb or hindlimb. They conclude that the type of skin may influence the central connections of DRG neurons that by chance innervate it in development.

After metamorphosis anurans may respond to an irritating cutaneous stimulus by wiping the site one or more times with a limb. The back is wiped with the hindlimb, the belly with the forelimb (Miner, 1956). The response may be easily elicited following spinal transection, as is also the case for the scratching reflex in mammals (Sherrington, 1910; Berkinblit, Deliagina, Feldman, Gelfand and Orlovsky, 1978). Stimulation of back skin transplanted prior to metamorphosis onto the belly may evoke misdirected wipes by the hindlimb and belly skin transplanted to the back may, upon stimulation, give rise to misdirected wipes by the forelimb (Miner, 1956; Baker, 1968; Jacobson and Baker, 1969). The result could represent respecification of the central connections of sensory neurons that unselectively reinnervated the skin (Miner, 1956; Baker, 1968; Jacobson and Baker, 1969) or it could be due to selective death of prespecified neurons that reinnervated mismatching skin (Gaze, 1970; Baker, 1978; Baker, Corner and Veltman, 1978). The topographic relations of DRG neurons projecting to either back or belly skin are unaltered in skin-rotated frogs, however in operated animals there is an overall shift in neuron positions which suggests some cell death occurs after the skin transplantation (Baker, Corner and Veltman, 1978).

Both the ventral and dorsal cutaneous sensory neurons terminate centrally in overlapping arbours that do not extend beyond thoracic segments, and no central rearrangement is observed in skin-rotated frogs that display misdirected wipes (Székely, Matesz, Baker and Antal, 1982).

Most of these studies have led to the conclusion that once DRG neurons have formed peripheral terminals, they are subjected to retrograde determinants, which in some way influence their connections within the CNS.

1.5 ORIGINS OF NEURON DIVERSITY

The preceding observations raise the central problem in developmental biology, the origins of cellular diversity. For the nervous system this problem was brought into the realm of chemical embryology largely by the work of Sperry (1963). In the early 1940s the two major hypotheses for the diversity of neural connections were contact guidance along orientated substrates (Weiss, 1934, 1945), and experience in the form of repetitive usage, which stabilized synapses. In reaction to those views Sperry (1941, 1942, 1943 a, b, 1945 a, b, 1947, 1963, 1965) proposed that selective nerve connections arise according to a biochemically determined plan, irreversibly imposed during embryonic development by the various nerve endings in the body, then extending to the higher centres, and, in the form of learning, imposed by the external environment via the sensory organs. The basic tenet of the theory, that a biochemical diversity underlies the specificity of neuron

connections, is now widely accepted, however the bases and origins of this diversity remain largely unknown.

The capacity of the early neural plate to restore its overall pattern following removal, rotation or translocation of its parts could only arise by interactions between the constituent cells. Such a group of cells, or cell surface, acting as a unit, is known as a morphogenetic field (see Wolpert, 1969). The two major properties of morphogenetic fields are those of divergence or discontinuity between neighbouring cells along the field boundaries, and intercellular communication within the field (French, Bryant and Bryant, 1976). Neither of these properties are presently understood in molecular terms, if indeed that is possible (see below).

Many studies of cell communication within morphogenetic fields have led to the suggestion that some form of polarity, or gradient is maintained between sources and sinks located on opposite boundaries of the field (Lawrence, Crick and Munro, 1972; Summerbell, Lewis and Wolpert, 1973; McCabe and Parker, 1976; Wolpert, 1978). It has also been suggested that the molecular basis of polarity might be chemical diffusion (Turing, 1952; Crick, 1970; Gierer and Meinhardt, 1972), or active transport of molecules or ions (Lawrence, 1966, 1969). The cells in the neural plate, and between the neural plate and lateral ectoderm, are electrically coupled (Warner, 1973) probably via subluminal gap junctions (Decker and Friend, 1973; Wilson and Finta, 1980) at early stages. The membrane potential of cells in the developing neural plate increases in relation to the surrounding ectoderm due to a rise in activity

of the sodium pump (Blackshaw and Warner, 1976 b; Messenger and Warner, 1979). The current resulting from this increasing potential difference might establish electrophoretic movement of charged particles, such as proteins (Wada and Nakamura, 1981) from cell to cell (Warner, 1981), or govern protein phosphorylation levels. (Goodwin and Pateromichelakis, 1979).

The problem of diversification of neighbouring cells in the formation of new field boundaries has undergone radical revision in the past decade, in disciplines far removed from molecular biology. Although these views have not as yet gained wide acceptance, they are introduced as a cautionary note here. As an example, the site of invagination of the embryo during gastrulation is the blastopore, and transplantation of the dorsal lip of the blastopore to a second host resulted in the formation of an almost complete secondary embryo (Spemann, 1938; Waddington, 1936). For several decades a search was conducted for the chemical 'inducer' assumed to be released from this organization centre. It eventuated that almost any explanted tissue was competent, as were unspecific chemicals such as methylene blue, and weak alkali or acids (Holtfreter, 1947). Thus almost any disturbance within the organizing region could lead to formation of a second field. (Such a non-specific reaction has recently been reported for retinoic acid on the vertebrate limb - Tickle, Alberts, Wolpert and Lee, 1982).

This raised the possibility that random fluctuations within an otherwise homogeneous layer of cells, if amplified, might lead to

a breakdown of stability, and progression to a new state of order. Transitions of this type, arising due to intermediate causes such as growth, would remove the necessity for chemical inducing agents, and were first formally examined by Turing (1952), followed by Gierer and Meinhardt (1972). Phase transitions have received considerable treatment as 'catastrophe' theory in topology (Thom, 1972), and in non-equilibrium thermodynamics (Nicolis and Prigogine, 1977). Whereas chemical diffusion within a field could be described in terms of reaction rates that were linear functions of the chemical concentrations, behaviour at the onset of instability was non linear, requiring stochastic methods (Turing, 1952). Although the time of transition was then more or less indeterminate, the number of possible outcomes was generally much smaller than the number of fluctuations giving rise to them, and they could often be classified topologically (Thom, 1972). Cell division (Cattaneo, Quastler and Sherman, 1961; Burns and Tannock, 1970; Smith and Martin, 1973; Shields, 1977; Shields and Smith, 1977; Shields, 1978; Brooks, Bennett and Smith, 1980; Brooks, Smith, Bennett and Richmond, 1981), and supernumerary limb production (Turner, 1981) have subsequently been identified as random transitions of fixed probability. These findings raise the possibility that for some irreversible aspects of development it may be difficult, or impossible to determine the immediate causes of particular events, until after they have occurred.

1.6 DEVELOPMENT OF THE ACTION POTENTIAL

Ultimately the neuroblasts become neurons, grow an axon and develop other properties of adult neurons. The capacity to produce action potentials develops after the transition from neuroblast to neuron (Spitzer and Lamborghini, 1976, Messenger and Warner, 1979). The voltage dependent outward channel, which gives rise to the falling phase of the action potential, appears first (Warner, 1973). The inward current responsible for the rising phase then develops. The inward current is first carried by calcium ions, then by calcium and sodium, and finally by sodium ions alone (Spitzer and Lamborghini, 1976; Spitzer, 1979 a; a review by Spitzer, 1979 b). These changes may be related to structural changes in the cell, since sodium and calcium spikes may be selectively reduced by disruption of microfilaments, or microtubules, respectively (Fukuda, Kameyama and Yamaguchi, 1981).

1.7 OUTGROWTH OF MOTOR AXONS TO THE LIMB

The transition from neuroblast to neuron is followed by the outgrowth of motor axons to the limb. The motor axons grow laterally to leave the spinal cord at the same rostrocaudal level as the soma (see Figure 3 in Lewis, Chevallier, Kieny and Wolpert, 1981). They first grow in the clefts between the myotomes in order to reach the limb. The number of myotome clefts therefore determines the number of segmental nerves supplying the limb (Detwiler, 1936). Addition or removal of somites results in a corresponding addition or reduction in the number of segmental nerves to the limb or fin

(Detwiler, 1934). This number may normally be over 40 in some fish species, or as low as 1, in the supply to the forelimb of some amphibia. According to the relative positions of the limb, somites and cord, the total number of limb nerves and the segmental contribution may vary between individuals of the same species (Sherrington, 1892; Romanes, 1951; Cruce, 1974 a).

The motor axons initially take the same course as the primary axons that innervate the myotome muscle fibres (Taylor, 1943; Prestige and Wilson, 1980). Following early limb bud removal in the chick, the axons grow as far as the base of the notocord, and form a neuroma (Oppenheim, Chu-Wang and Maderdrut, 1978). This suggests that the initial outgrowth is not dependent on the presence of the limb. To reach the limb bud the axons leave the myotome clefts and pass through the body wall. In amphibians, a specialized strand of connective tissue forms in this region (Prestige and Wilson, 1980).

In the vicinity of the limb the axons seem to be attracted to their target. Limbs transplanted to a more caudal location at this time acquire the normal complete set of segmental nerves (Detwiler, 1920 a). These may include one or more nerves from thoracic segments.

1.8 DEVELOPMENT OF THE LIMB AND LIMB NERVE PLEXUS

The motor axons reach the base of the limb when it is a small bud, at stage 49 (Nieuwkoop and Faber, 1967) in *Xenopus*, and stage 21 (Hamburger and Hamilton, 1951) in the chick embryo (Bennett, Davey and Uebel, 1980). The parts of the limb subsequently differentiate in sequence (see Wolpert, Lewis and Summerbell, 1975). The proximal structures are the first to be determined, followed in succession by more distal structures, concluding with the digits. Differentiation follows the same sequence (Kieny, 1977).

The axons enter the limb bud at stage 50 in *Xenopus* (Lamb, 1974) and stage 23 in the chicken (Bennett, Davey and Uebel, 1980), before cleavage of the muscle masses. Within the limb bud the axons are not accompanied by Schwann cells and do not grow in parallel (Prestige and Wilson, 1980). The major limb nerve branches become recognizable at stages 51-52 in the amphibian, although some axons ramify widely through the mesenchyme (Taylor, 1943). Myotubes form at stage 53 (Muntz, 1975).

The segmental pattern of the nerves is obscured at the base of the limb, by the formation of the limb nerve plexus. A limb grafted alongside the normal member results in the formation of an additional plexus that is characteristic of the type of limb (Hamburger, 1939 b; Morris, 1978). Thoracic or cervical spinal cord transplanted to the brachial level will innervate the wing and form a brachial plexus (Wenger, 1950; Straznicky, 1963). It was suggested by Bennett, Davey and Uebel (1980) that the plexus

arises by a sequential spatial and temporal attraction of axons as they arrive at the base of the limb, by the differentiating dorsal and ventral muscle masses.

1.9 SOMATOTOPIC ORGANIZATION OF LIMB MOTONEURONS

The topographic relations of each muscle in the limb are reflected in the relative position of its motoneurons in the spinal cord. This has been demonstrated in urodeles (Székely and Czéh, 1967), anurans (Cruce, 1974 a; Lamb, 1976), chickens (Landmesser, 1978 a; Pettigrew, Lindeman and Bennett, 1979; Hollyday, 1980 b), cats (Rexed, 1952; Romanes, 1964), mice (McHanwell and Biscoe, 1981), rats (Iles and Nicolopoulos, 1981) and humans (Sharrard, 1955). This somatotopic organization is basically similar in all vertebrate species with a recognizable LMC. The motoneuron somas projecting to each muscle are grouped in separate pools that may overlap at a given rostrocaudal level. There is also correspondence, with some exceptions, between the rostrocaudal and mediolateral locations of motoneuron somas, and the proximo-distal and dorsoventral regions of the limb. Most of the exceptions arise by morphogenetic movements of muscle primordia (Landmesser, 1978 a; Hollyday, 1980 b), and muscle loss (Lance Jones, 1979) after connections have formed.

There are several reports of somatotopic organization within the projection of a single motor pool to a muscle. The most rostral third of the cat gastrocnemius motoneuron pool contains a higher ratio of large to small alpha cells, which project to the larger motor units located in the dorsal region of the muscle (Burke,

Strick, Kanda, Kim and Walmsley, 1977; see also Swett, Eldred and Buchwald, 1970). Regional differences in the territories of different segmental nerves supplying the same muscle have also been noted in rats (Browne, 1950) and frogs (Boyd, 1926; Boer, 1927; Bennett and Lavidis, 1982), however the somatotopic organization does not necessarily extend to the level of individual neurons.

Although vertebrate species differ widely in terms of limb motor functions (for locomotion see Grillner, 1975), these differences are not reflected in the somatotopic organization of their motoneurons, which is conservative.

1.10 NEURON DEATH DURING DEVELOPMENT

1.10.1 Neuron Death Induced by Target Ablation

An early neuroembryological finding was that removal of a target during a particular period of development resulted in hypoplasia of the associated nervous centre (Shorey, 1909). This was later attributed to the death of many of the motoneurons, not a failure of their precursor neuroblasts to proliferate or differentiate (Hamburger, 1958; Hughes, 1961). The phenomenon has been described in a number of neuron populations in a wide variety of species (see Table 2).

In the case of the lateral motoneurons, the assembly, initiation of axon outgrowth (Prestige, 1970, 1976; Oppenheim, Chu-Wang and Maderdrut, 1978) and development of acetylcholinesterase and

TABLE 1.1 REPORTED NEURON LOSSES DURING NORMAL DEVELOPMENT

TABLE 1.2 REPORTED NEURON LOSSES FOLLOWING TARGET ABLATION

TABLE 1.3 REPORTED REDUCTION OF NEURON LOSSES FOLLOWING TARGET ENLARGEMENT

TABLE 1

REPORTED NEURON LOSSES DURING NORMAL DEVELOPMENT

	STAGES*	% LOST**	REFERENCE
INVERTEBRATES			
Insect optic lobe	larva-pupa	-	(Nordlander and Edwards, 1968)
Hermit crab pleopod motoneurons	larva-adult	-	(Chapple, 1977)
Insect abdominal ganglion	embryo	-	(Bate, Goodman and Spitzer, 1981)
VERTEBRATES			
AMPHIBIA			
Xenopus laevis (clawed toad)			
Lumbar LMC	53-60	70	(Hughes, 1961; Prestige, 1967)
Brachial LMC	53-60	70	(Fortune and Blackler, 1976)
Thoracic DRG	53-60	70	(Prestige, 1965)
Lumbar DRG	53-66	70	(Prestige, 1965)
Rohon-Beard neurons	?-53	100	(Hughes, 1957)
Hyla punctatissima			
Lumbar LMC	premet	80	(Hughes, 1963)
Brachial LMC	premet	80	(Hughes, 1963)
Bufo marinus			
Lumbar LMC	premet	55	(Hughes, 1968)
Rana pipiens			
Lumbar LMC	XI-XXV	60	(Pollack, 1969)
Brachial LMC	XI-XXV	60	(Pollack, 1969)
Lumbar DRG	X-XX	53	(Bibb, 1978)
Rana temporaria			
Lumbar LMC	X-XXV	80	(Race and Terry, 1965)
E. martinicensis			
LMC	premet	67	(Hughes, 1968)
DRG	premet	-	(Hughes and Egar, 1972)
E. ricordii			
Lumbar DRG	premet	-	(Hughes, 1959)
Thoracic DRG	premet	-	(Hughes, 1959)
Brachial DRG	premet	-	(Hughes, 1959)
Rohon-Beard neurons	premet	100	(Hughes, 1959)
Limnodynastes peronii			
Thoracic DRG	14-24	-	(Bennett and Lai, 1981)

TABLE 1 continued.

REPORTED NEURON LOSSES DURING NORMAL DEVELOPMENT	STAGES*	% LOST**	REFERENCE
AVES			
Gallus domesticus (chicken)			
Lumbar LMC	d6.5-9.5	40	(Hamburger, 1975)
Brachial LMC	d6 -21	60	(Oppenheim and Majors-Willard, 1978)
Brachial DRG	d4.5-9	-	(Carr and Simpson, 1978)
Thoracic DRG	d5 -7	-	(Hamburger and Levi-Montalcini, 1949)
Thoraco-lumbar preganglionic	d8 -10	-	(Oppenheim, Maderdrut and Wells, 1982)
Vagus dorsal motor nucleus	d8 -12	21	(Wright, 1981)
Mesencephalic nucleus of trigeminal	d9 -13	75	(Rogers and Cowan, 1972)
Inferior olive	d16 -20	30	(Armstrong and Clarke, 1979)
Isthmo-optic nucleus	d10 -17	60	(Cowan and Wenger, 1978)
Purkinje cells	d17 -	-	(Fritzsch, 1979)
Ciliary ganglion	d9 -13	50	(Landmesser and Pilar, 1974)
Retinal ganglion cells	d10 -18	20	(Hughes and McLoon, 1979)
Optic tectum	d10 -11	-	(Cantino and Daneo, 1972)
MAMMALIA			
Didelphis virginiana (Opossum)			
Brachial LMC	d12 -	-	(Hughes, 1973)
Lumbar LMC	d28 -	-	(Hughes, 1973)
Brachial DRG	d13.5-	-	(Hughes, 1973)
Mus musculus (Mouse)			
Lumbar LMC	pd2 -12	30	(Romanes, 1946)
LMC	d11 -15	-	(Flanagan, 1969)
Rattus rattus (Rat)			
Brachial LMC	pd 1 - 7	45	(Nurcombe, McGrath and Bennett, 1981)
Brachial LMC	pd10 -200	75	(Tada, Ohshita, Yonenobu, Ono, Satoh and Shimizu, 1979)
Puchinje cells	pd10 -	-	(Fritzsch, 1979)
Retinal ganglion neurons	pd 2 - 10	35	(Potts, Dreher and Bennett, 1982)
Optic tectum	pn 1 -	-	(Arees and Angström, 1977)
Mesocricetus auratus (Hamster)			
Retinal ganglion neurons	pd 1 -10	49	(Finlay, Berg and Senzebelaub, 1982)
Superior collicus	pn 1 - 8	19-38	(Finlay, Berg and Senzebelaub, 1982)

(1) For a synopsis of amphibian normal tables of development see Nieuwkoop and Faber, 1967.

(2) d = days post-fertilization
pd = days post-natal
premet = premetamorphosis

TABLE 2

REPORTED NEURON LOSSES FOLLOWING TARGET ABLATION

	STAGES *	% LOST **	REFERENCE
INVERTEBRATES			
Sarcophaga bullata	pre-post embryonic	29	(Chiarodo, 1963)
VERTEBRATES			
AMPHIBIA			
Xenopus laevis (Clawed toad)			
Lumbar LMC	54-57	100	(Prestige, 1970)
Brachial LMC	54-57	80	(Fortune and Blackler, 1976)
Lumbar DRG	53-55	50	(Prestige, 1967)
Amblystoma			
Lumbar LMC	limb bud	50	(Stultz, 1942)
DRG	limb bud	-	(Shorey, 1909)
Bufo americanus			
LMC	premet	-	(Shorey, 1909)
Rana pipiens			
Lumbar LMC	premet	82	(Beaudoin, 1955)
E. martinicensis			
Lumbar DRG	d5 -10	-	(Hughes, 1964)
AVES			
Gallus domesticus (Chicken)			
Brachial LMC	d4 -10	22-61	(Hamburger, 1934)
Lumbar LMC	d2 -9	90	(Hamburger, 1958)
DRG	d5 -6	-	(Hamburger and Levi-Montalcini, 1949)
Isthmo-optic nucleus	d6-19	100	(Cowan and Wenger, 1968)
Trochlear nucleus	d11-26	80	(Cowan and Wenger, 1967)
Ciliary ganglion	d9-11	92	(Landmesser and Pilar, 1974)
Retinal ganglion neurons			(Hughes and Lavelle, 1975)
MAMMALIA			
Ovis aries (Sheep)			
DRG	in utero	11-93	(Barron, 1945)
Mus musculus (Mouse)			
Lumbar LMC	pd1 -7	40-50	(Romanes, 1946)
Rattus rattus (Rat)			
Lumbar LMC	d18-pd25	-	(McLennan and Hendry, 1981)
DRG	d15-pd1	-	(Hall and Schneiderhan, 1945)

*For a synopsis of amphibian normal tables of development see Nieuwkoop and Faber, 1967.

**d = days post-fertilization
pd = days postnatal
premet = premetamorphosis

TABLE 3

REPORTED REDUCTION OF NEURON LOSSES FOLLOWING TARGET ENLARGEMENT

	STAGES*	% INCREASE**	REFERENCE
INVERTEBRATES			
Aeshna cyanea			
Optic lobe	Larva	11	(Mouze, 1978)
AMPHIBIA			
Amblystoma			
Thoracic DRG	Limb bud	25-60	(Detwiler, 1920)
Limb DRG	Limb bud	-	(Carpenter, 1932)
Discoglossus pictus			
Limb DRG	Limb bud	-	(May, 1933)
Rana			
Limb DRG 8,9	Limb bud	71	(Buecker, 1945)
Lumbar LMC	Limb bud	23	(Buecker, 1945)
Rana pipiens			
Brachial LMC			(Pollack, 1969)
DRG 9	V-XX	60	(Bibb, 1978)
Xenopus laevis (Clawed toad)			
Lumbar LMC	49-54	4-28	(Hollyday and Mendell, 1975)
Lumbar LMC	51-53	0- 7	(Rubin and Mendell, 1980)
Thoracic DRG	49-54	54	(Hollyday and Mendell, 1975)
Lumbar DRG	51-53	3-46	(Rubin and Mendell, 1980)
AVES			
Gallus domesticus (Chicken)			
Lumbar LMC	d2.5-11	11-28	(Hollyday and Hamburger, 1976)
Limb DRG	d2.5- 9	15-200	(Hamburger, 1939)
Trochlear nucleus	d2 -19	19-62	(Boydston and Sohal, 1979)
Isthmo-optic nucleus	d2 -19	27-41	(Boydston and Sohal, 1979)
Ciliary ganglion	d2 -15	8-27	(Narayanan and Narayanan, 1978)
Accessory occulomotor nucleus	d2 -15	9-33	(Narayanan and Narayanan, 1978)
MAMMALIA			
Mus musculus (Mouse)			
LMC (hyperdactyly)	prenatal	-	(Tsang, 1939)

*For a synopsis of amphibian normal tables of development see Nieuwkoop and Faber, 1967.

**d = days post-fertilization
pd = days postnatal

choline acetyltransferase enzyme systems (Oppenheim, Chu-Wang and Maderdrut, 1978) is not dependent on the presence of a limb. The sensitivity to limb amputation begins at a time when the first limb movements occur (Hamburger, 1934; Prestige, 1967 a) and most motoneurons have axons in the vicinity of the limb (Prestige and Wilson, 1974; Prestige, 1976; Chu-Wang and Oppenheim, 1978 b; Oppenheim, Chu-Wang and Maderdrut, 1978). Within the spinal cord, dendrites of motoneurons in different pools overlap extensively at each rostrocaudal level (frog - Stensaas and Stensaas, 1971; Székely, 1976; cat - Romanes, 1964). Furthermore each limb-moving segment of the amphibian (Székely and Czéh, 1976) or cat spinal cord (Grillner and Zangger, 1979) is capable of delivering a complete locomotor cycle in terms of alternating flexor and extensor activity. These observations suggest that the somatotopic organization bears only a coincidental relationship to functional organization, which is based largely on local neuron interactions at each spinal cord level (Cruce, 1974 a).Similarly in invertebrates (Stuart, 1970; Bentley, 1970; Davis, 1971; Burrows and Hoyle, 1973) it has been concluded that the regular positions of motoneuron somas bears no close relation to the motor output pattern (Kennedy and Davis, 1977).

A number of workers have assigned a developmental significance to somatotopic organization. The strong correlation between motoneuron positions in the spinal cord and the precursor muscle masses in the developing limb (Landmesser, 1978 a; Hollyday, 1980 b) could serve to at least approximately connect each motoneuron with the muscle for which its central connections were most appropriate (Hughes, 1968;

Cruce, 1974; Horder, 1978; Landmesser, 1978 a; Hollyday, 1980 b). This would require motor axons to maintain some order in growing from the cord to the limb and a number of experiments suggest that this occurs (Stirling and Summerbell, 1979; Summerbell and Stirling, 1981).

Horder (1978) argues that somatotopic organization coupled with morphogenetic events in the limb are sufficient to match motoneurons and muscles exactly. Others suggest that the correspondence is not precise and that exact matching is achieved by pathway selection by motor axons (Lance Jones and Landmesser, 1980, 1981 a, b) or by the death of motoneurons that form 'erroneous' connections (Pettigrew, Lindeman and Bennett, 1979).

It was also noted by Shorey (1909) that removal of the limb bud in *Amblystoma*, *Rana* or *Bufo* resulted in hypoplasia of the associated limb ganglia. This observation has been confirmed for the DRG in a wide variety of vertebrates (see Table 1). The hypoplasia was later attributed to both neuron death, and a failure of neuroblasts to proliferate and differentiate (Hamburger and Levi-Montalcini, 1949). Immediately following limb amputation there is a decline in the number of degenerating cells. Autoradiographic labelling of ganglia following wing bud ablation in the chick between days 4.5 to 9.5 suggest that this response is due to increased proliferation by glial cells, not neuroblasts (Carr and Simpson, 1978 a, b). During this time at least some neurons have axons in the limb bud, as evident by labelling of the soma following horseradish peroxidase injection into the limb bud (Oppenheim and Heaton, 1975), and electron microscope studies of development of the spinal roots (Prestige and Wilson, 1980).

In general not all limb motoneurons die following target ablation (see Table 1), but in the case of a unilateral limb amputation some motor axons then project to the contralateral limb (Lamb, 1980) and removal of both limbs results in 100% limb motoneuron death (Lamb, 1981, a). If the *Xenopus* hind limb bud is amputated at stage 54, when limb movements are first evident (Hughes and Prestige, 1967) a large loss of motoneurons occurs within 3 days. The same operation at stage 57 results in many neurons producing a chromatolytic reaction, followed by a slow loss of reacting neurons over several weeks (Prestige, 1967 a). These observations suggest that *all* the motoneurons during normal maturation become dependent in some way on their peripheral connections, although only a percentage normally survive (Prestige, 1970).

1.10.2 Incidence of Neuron Death During Normal Development

Neuron death also occurs during normal development, in many regions of the central and peripheral nervous system (see Table 1). It may take two forms. Where the loss is total it is associated with the complete developmental regression of a target. This may occur in developing neurons, as with the early loss of the Rohon-Beard neurons in larval amphibia (Hughes, 1957), or it may involve mature neurons, as with the loss of the Mauthner neurons in association with regression of the tail in amphibian metamorphosis.

In the second form of neuron death, some neurons in the population survive while others die. Following early observations of degenerating neurons in the frog (Barbieri, 1905) and chicken spinal

cord (Collin, 1906), inventories of the loss of limb motoneurons during development showed that it exceeded 50% of the total produced (Hughes, 1961; Hamburger, 1975). At its peak about 5% of the motoneurons at any one time showed signs of degeneration (Hughes, 1961). The natural death of neurons coincides with the developmental stages during which sensitivity to target amputation is at a maximum. The histological events are similar during natural and induced death (Chu-Wang and Oppenheim, 1978 a), which suggests that naturally occurring neuron death may also reflect a failure of some neurons to form or maintain their peripheral connections.

Similarly, during the period of maximum sensitivity to target ablation, over 50% of the DRG neurons die during normal development (see Table 2). This period corresponds to stages at which there is naturally occurring motoneuron death. The skin structures are also embryologically determined at these stages (Cole, 1922; Helf, 1931; Herrick, 1932). Enlargement of the periphery results in DRG hyperplasia which often exceeds that of the motor centres in relative terms (see Table 3). This may result from a reduction in naturally occurring neuron losses.

1.10.3 Evidence for Peripheral Trophic Factors

Prestige (1967 a) suggested that from the time of muscle innervation the motoneurons are dependent on a trophic factor derived by retrograde transport from the muscle fibres.

Studies in vitro have shown that media conditioned by contact with heart or skeletal muscle cells contain factors that promote survival and/or neurite outgrowth from ciliary ganglion cells (Nishi and Berg, 1981), tadpole spinal cord (Pollack, 1980) and motoneurons in cultures of dissociated spinal cord (Bennett, Lai and Nurcombe, 1980). It is not yet known whether any of these putative factors are involved in normal development. It should be noted that neurons in tissue culture are not subject to competition as in normal development.

On the basis of studies of the effects of limb amputation on the neurons of the DRG, Prestige (1967 b) postulated that during their normal maturation the neurons become dependent on a 'maintenance factor' derived from the periphery and transported to the cell body by retrograde axonal flow. It has been shown that the polypeptide hormone 'nerve growth factor' (NGF) is essential for survival and development of sensory and sympathetic neurons (reviewed by Levi-Montalcini and Angeletti, 1968). The injection of NGF into early chick embryos may greatly reduce the naturally occurring loss of both large and small diameter DRG neurons (Hamburger, Brunso-Bechtold and Yip, 1981), and injection of iodine-labelled NGF into the chick embryo leg results in uptake to the DRG (Brunso-Bechtold and Hamburger, 1978).

1.10.4 Effects of Transmission Blockade

There have been several reports of a reduction, or a delay in the normally occurring death of motoneurons following neuromuscular blockade (Laing and Prestige, 1978; Pittman and Oppenheim, 1978; Oppenheim and Majors-Willard, 1978; Olek, 1980). The blockade may be effective either presynaptically, using botulinum toxin (Pittman and Oppenheim, 1978) or postsynaptically, using curare, (Laing and Prestige, 1978; Pittman and Oppenheim, 1978). Removal of the block resulted in a delayed period of motoneuron death with neuron numbers declining to normal levels. When performed at a late stage, the motoneurons survived in unusually high numbers (Olek, 1980).

On the basis of these results it has been suggested that neuron death may be regulated in some manner by synaptic and/or muscle activity (Pittman and Oppenheim, 1978). It has also been shown that short-term continuous electrical stimulation of hind limb musculature or nerve trunks may cause a significant increase in motoneuron death (Oppenheim and Núnez, 1982).

An alternative suggestion (Mark, 1980) for the reduction of neuron death is that the profusion of endplates and motor nerve sprouts in the presence of curare (Oppenheim and Majors-Willard, 1978) or botulinum toxin (Giacobini, Filogamo, Weber, Boquet and Changeux, 1973) in effect greatly enlarges the available target, and so enhances survival.

Motoneuron death has been reported in tadpoles raised under chlorbutanol anaesthesia (Olek and Edwards, 1980), and such animals develop proper limb motor co-ordination (Harrison, 1904), however it is likely in these cases that there is at least a residual release of transmitter at the neuromuscular junctions.

1.10.5 Formation of Central Connections

A synopsis of the central events accompanying neuron death in three commonly studied species is given in Figures 1(a), (b), (c) respectively.

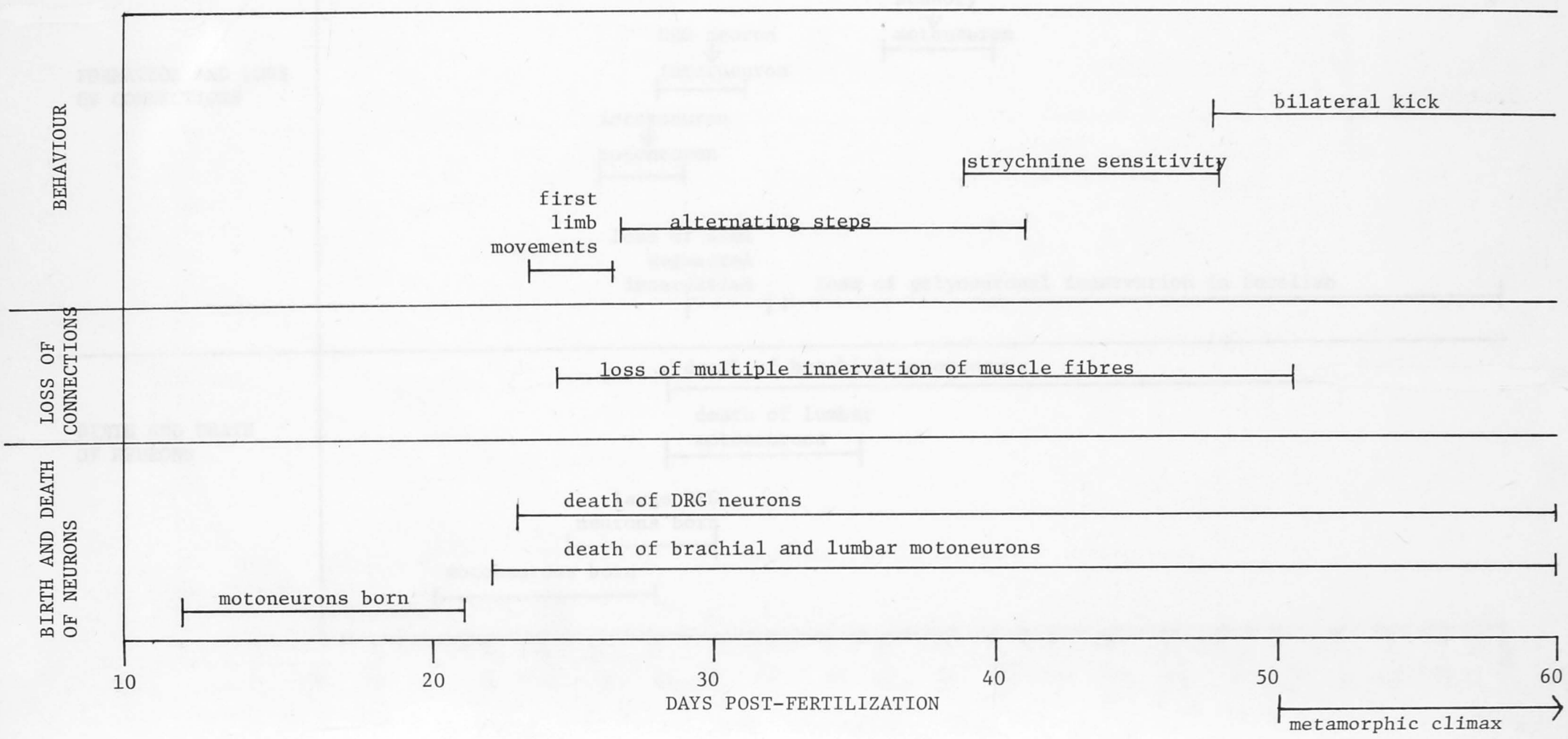
In the chick lumbosacral cord the first synapses made onto the motoneurons are evident at day 5 (Oppenheim, Chu-Wang and Foelix, 1975) when functional neuromuscular junctions have formed (Landmesser and Morris, 1975). They probably derive from commissural and association interneurons (Oppenheim, Chu-Wang and Foelix, 1975). On days 7.5 - 8, afferent collaterals from the dorsal funiculus contact commissural and association interneurons, to complete the first reflex arcs. Reflexes in response to tactile stimulation can then be elicited (Windle and Orr, 1934). There is therefore a retrograde sequence of synapse formation onto the motoneurons, which in the chick may give rise to co-ordinated motor output, in terms of flexor and extensor bursts in the 7 day old embryo (Bekoff, Stein and Hamburger, 1975). A similar retrograde sequence has been observed in embryonic mouse spinal cord (Vaughn and Grieshaber, 1973; Vaughn, Henrikson, Chernow, Grieshaber and Wimer, 1975; Vaughn, Sims and Nakashima, 1977).

FIGURE 1 (a) DEVELOPMENT OF LIMB INNERVATION IN *Xenopus laevis*

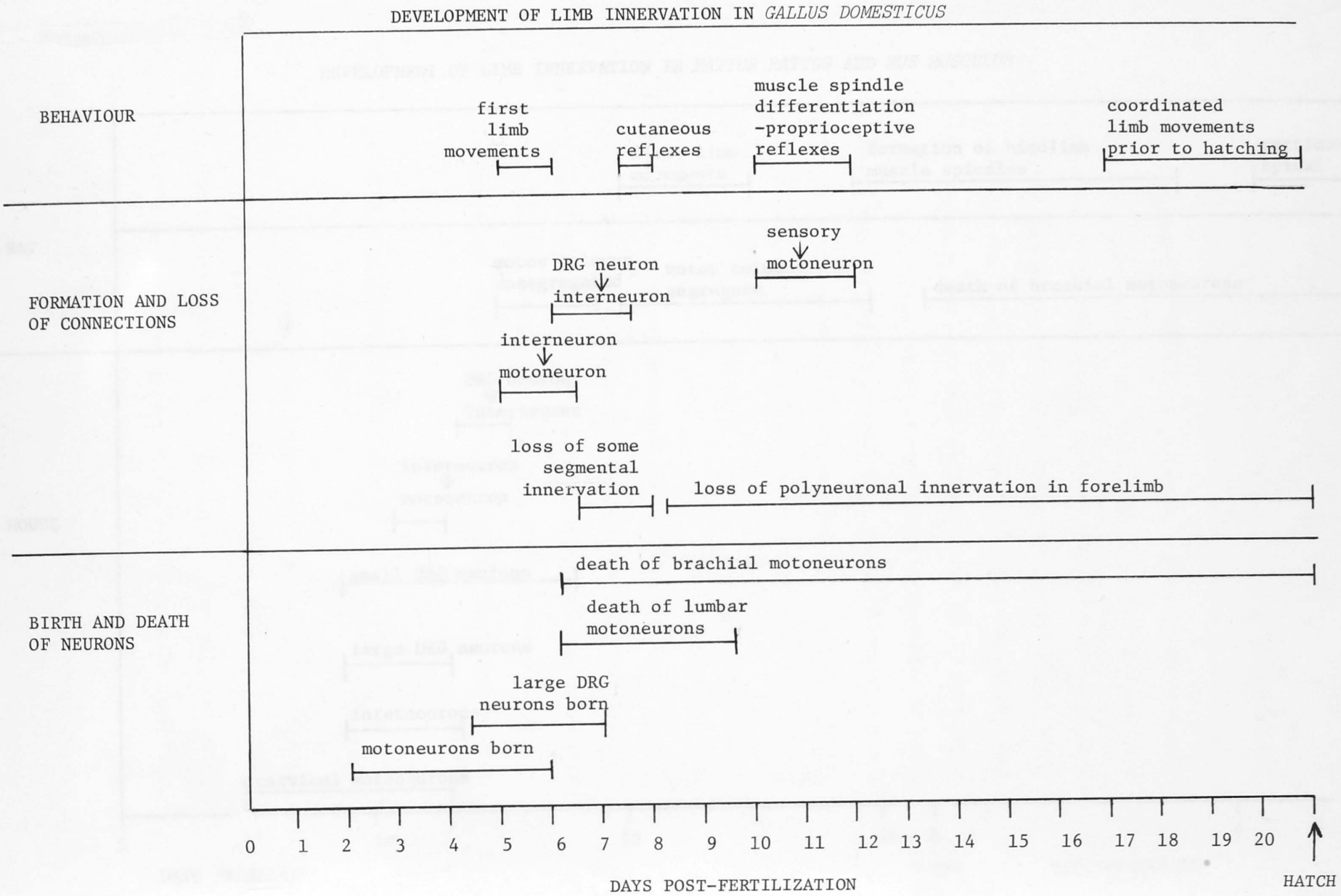
(b) *Gallus domesticus*

(c) *Rattus rattus* and *Mus musculus*

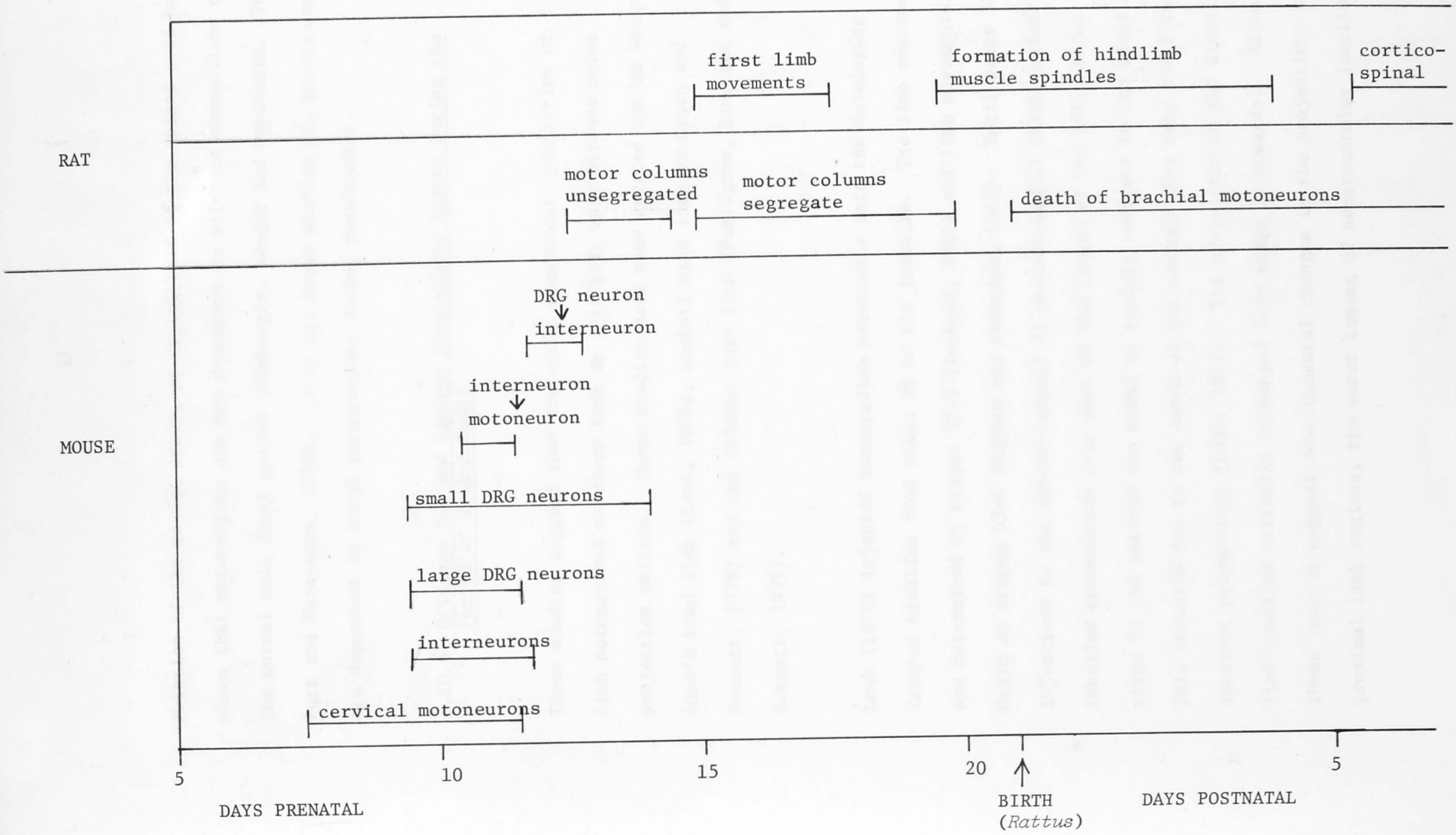
DEVELOPMENT OF LIMB INNERVATION IN *XENOPUS LAEVIS*



Xenopus laevis


DEVELOPMENT OF LIMB INNERVATION IN GALLUS DOMESTICUS
BEHAVIOUR
first limb movements
cutaneous reflexes
muscle spindle differentiation -proprioceptive reflexes
coordinated limb movements prior to hatching
FORMATION AND LOSS OF CONNECTIONS
sensory
motoneuron
DRG neuron
interneuron
interneuron
motoneuron
loss of some segmental innervation
loss of polyneuronal innervation in forelimb
BIRTH AND DEATH OF NEURONS
death of brachial motoneurons
death of lumbar motoneurons
large DRG neurons born
motoneurons born
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
DAYS POST-FERTILIZATION
HATCH

DEVELOPMENT OF LIMB INNERVATION IN *RATTUS RATTUS* AND *MUS MUSCULUS*



Ablation of the neural crest, or dorsal half of the spinal cord has shown that motoneurons are not dependent on afferent connections during the normal cell death period (Hamburger, Wenger and Oppenheim, 1966; Burt and Narayanan, 1970). It is not known whether the motoneurons are dependent on local interneurons during development.

1.10.6 Evidence for and Against Somatotopic Errors During the Period of Neuron Death

Three studies suggest that the adult somatotopic projection of limb motoneurons develops from an initially more diffuse motor projection pattern. These studies have been carried out on *Xenopus laevis* hind limb (Lamb, 1976), axolotl hind limb (McGrath and Bennett, 1979) and the chicken fore limb (Pettigrew, Lindeman and Bennett, 1979).

Lamb (1976) injected horseradish peroxidase into the developing *Xenopus* hindlimb from stage 50 to the juvenile. The limb regions are determined by stages 52-3 (Tschumi, 1957) and limb movements begin at stages 53-4 (Hughes and Prestige, 1967). Until stage 53, injections to the antero-lateral or postero-medial thigh regions labelled motoneurons over most of the length of the LMC. After stage 53 the pattern was found to rapidly contract to the adult form. This occurred due to the death of the neurons that make the first aberrant projections (Lamb, 1977). The projection to the distal limb remained virtually unchanged from stage 52 onwards. Although these results suggest developmental changes in the projections to proximal limb regions, the exact timing of neuromuscular junction

formation is uncertain, and it is not known whether the aberrant projections form functional connections in the limb.

Using a different experimental approach, McGrath and Bennett (1979) recorded end plate potentials in six sectors of the flexor muscle mass of the axolotl hindlimb in response to electrical stimulation of each spinal nerve in turn. At the earliest stage examined, spinal nerves 16 and 17 both projected to each muscle sector. By the time the muscle had grown from 1.5 mm length at this stage to 8.5 mm, the nerves had segregated their territories to form the adult pattern.

A similar study was performed in the biceps muscle of the chick forelimb (Pettigrew, Lindeman and Bennett, 1979). By measuring compound action potentials in the presumptive biceps muscle mass, it was found that axons projecting from segmental nerves 12 and 17 were lost between stages 26-29. Corresponding to this loss of axons, horseradish peroxidase injections to the biceps showed a corresponding reduction in the distribution of labelled motoneurons in the spinal cord segments. Similar results were obtained by electrophysiological and tension recordings in several other limb muscles. Intracellular recordings from stage 35 onwards (days 9-20) showed a loss of multiple innervation of synaptic sites that did not involve changes in the segmental innervation of the muscle fibre. It is not known whether a loss of multiple innervation occurs between stages 26-29.

A different conclusion concerning early motor projections has been reached in studies of development of chick hindlimb innervation (Landmesser and Morris, 1975; Landmesser, 1978 b). These studies used tension recordings and visual observations of contractions in embryos from stages 25 to 43 (Landmesser and Morris, 1975) and horseradish peroxidase injections to the primary muscle masses from stages 28 to 36 (Landmesser, 1978 b). The projection pattern was found to match that of the adult at all these stages.

The technical difficulties in this work should not be underestimated. The limbs of the embryo are extremely small and there is the possibility that diffusion of HRP from the injection site could give the mistaken impression that diffuse projections existed. A similar erroneous conclusion could arise in electrophysiological studies where electrical coupling of muscle fibres was not adequately taken into consideration.

One study suggests that cutaneous sensory neuron death is accompanied by changes in the topographic distribution of cells within the ganglia (Bennett and Lai, 1981 a, b). In the frog *Limnodynastes* at early stages the small and large diameter DRG neurons innervating dorsal or ventral skin are intermingled within the thoracic ganglia. At later stages the dorsal and ventral projecting neurons are segregated into two groups (Bennett and Lai, 1981 a) and this arises at least partly by death of the neurons making projections that do not conform to the adult pattern (Bennett and Lai, 1981 b).

1.10.7 Hypotheses as to the Significance of Neuron Death

There have been reports of an initially diffuse motor projection to the amphibian hindlimb (Lamb, 1976; McGrath and Bennett, 1979) and chicken forelimb (Pettigrew, Lindeman and Bennett, 1979) in development. It has been suggested that the adult somatotopic projection arises by the death of motoneurons that have inappropriate central connections for the action of the muscle in which they terminate (Hughes, 1968; Lamb, 1977; Pettigrew, Lindeman and Bennett, 1979). A cause of death may be competition between neurons for terminal sites in the periphery (Prestige, 1970), *and competition between sites for axons*, as is necessary to produce topographically ordered maps (Prestige and Willshaw, 1975).

There have also been reports suggesting that chick lumbar motor axons grow precisely to their matching hindlimb muscles, with no evidence of early diffuse projections (Landmesser and Morris, 1975; Landmesser, 1978 b). This correspondence could arise by a precise coupling of axon outgrowth and morphogenetic events in the limb (Horder, 1978). An alternative suggestion is that motor axons selectively grow to their appropriate muscles (Lance-Jones and Landmesser, 1980, 1981 a, b). This suggestion is based on the finding that motoneurons connect with their appropriate muscles following anterior-posterior reversal of 2 or 3 spinal cord segments (Lance-Jones and Landmesser, 1980) or small anterior-posterior shifts of segments (Lance-Jones and Landmesser, 1981 b) made prior to motor axon outgrowth.

When motor axons grow to a limb experimentally reversed about either the dorso-ventral (Morris, 1978; Summerbell and Stirling, 1981) or anterior-posterior axes (Stirling and Summerbell, 1979) however, they do not alter their pathways, and innervate atypical muscles. Similarly Lewis (1978) showed that in limbs with reduplicated distal parts the proximal brachial nerve subdivided to form ulnar and median nerves, and the median nerve again divided in the reduplicated section, to again form ulnar and median branches. These experiments therefore suggest that motor axon growth is unselective in terms of substrates.

If diffuse motor projections occur early in chick hindlimb development and were not detected, it is possible that the reversed projections following cord reversals arose due to non-selective outgrowth of motor axons, and subsequent death of motoneurons that formed mismatching connections (Mark, 1980). Landmesser (1980) suggests that motor axons may grow unselectively when in a foreign environment, and selectively when in the locality of their matching target.

An alternative suggestion to that of neuron death serving to eliminate mismatching connections is that it eliminates neurons produced in excess of the available target space (Cowan, 1973). There are a number of examples of a reduction in naturally occurring neuron losses following experimental enlargement of a peripheral target (see Table 3). It should be noted that in most cases the increase in survivors is not total, despite almost a doubling of the peripheral field size. There is also evidence however, that the peripheral target size does not influence the numbers of survivors.

Lamb (1980) amputated one *Xenopus* hindlimb and directed motor axons from both sides of the cord into the remaining limb prior to the neuron death period. The single limb was found to support a normal ipsilateral complement of motoneurons, and also a contralateral complement that in some cases was almost as large.

1.11 EVIDENCE FOR COMPETITION BETWEEN MATURE NEURONS AT THE NEUROMUSCULAR JUNCTION

It was noted by Detwiler (1920 a) that in urodele amphibia a transplanted forelimb rudiment that received innervation from the brachial plexus developed movements in co-ordination with the adjacent limb. Weiss (1922, 1937 a, b, c, d) repeated this experiment, using a differentiated limb, and also found that the limb developed co-ordinated, or 'homologous' movements. A myotypic 'Resonance' Principle was proposed (Weiss, 1926), whereby each muscle only responded to the particular excitations to which it was tuned. It was later suggested by Sperry (1941) that there was a rearrangement of central connections onto motoneurons reinnervating foreign muscles in the grafted limb. Efforts to show such changes in neonate mammalian spinal cord have continually failed (Sperry, 1941, 1942, 1945 a; Mendell and Scott, 1975). It should be noted that the physiological tests have only concerned possible changes in the primary afferent connections onto the motoneurons (Eccles, Eccles, Shealy and Willis, 1962; Mendell and Scott 1975) although these connections are not essential for the rhythmic generation of locomotor output (Grillner and Zangger, 1975).

The lack of adaptability evident in the spinal cord placed the burden of functional recovery on events in the periphery. There is now substantial evidence for selective reinnervation as the basis for the recovery of co-ordination in axolotl limbs upon regeneration of mixed motor nerves (Grimm, 1971; Cass, Sutton and Mark, 1973; Cass and Mark, 1975; Bennett and Raftos, 1977; Stephenson, 1979). This may depend at least partly on competitive events at the neuromuscular junctions.

In urodeles a limb motor nerve will form terminals in almost any denervated limb muscle (Yip and Dennis, 1976; Dennis and Yip, 1978; Wigston, 1979; Bennett, McGrath and Davey, 1979) and the quantal content may reach the same size as at the normal terminals (Dennis and Yip, 1978; Bennett, McGrath and Davey, 1979) but declines upon reformation of terminals in their vicinity by the original nerve. Decline in transmission is probably accompanied by regression of the presynaptic terminals (Dennis and Yip, 1978; Bennett, McGrath and Davey, 1979). There is evidence that competition between motor nerve terminals may take place on synapses separated by over 100μm on individual muscle fibres (Wigston, 1979).

A similar developmental regression of nerve terminals that do not correspond to the adult projection pattern to axolotl hindlimb flexor muscles has been observed by McGrath and Bennett (1979). It has been suggested that the phenomenon of synapse regression evident in urodeles may signify a process of widespread importance in the formation of specific neuron connections (Bennett, McGrath and Davey, 1979; reviewed by Mark, 1980).

In partially denervated mammalian skeletal muscle a second aspect of competition is evident. The remaining motor axons in that situation sprout, and innervate the denervated muscle fibres. Reinnervation results in reduction of the enlarged motor units to their normal sizes (Brown and Ironton, 1978; Slack and Hopkins, 1982). The reinnervation occurs at the same central innervated endplate on each muscle fibre (Brown and Ironton, 1978; Hopkins and Slack, 1981; Slack and Hopkins, 1982) and may lead to withdrawal of the sprouted terminals (Slack and Hopkins, 1982). Similar events are evident in the loss of polyneuronal innervation in the first few weeks after birth (see section 1.12).

The reinnervation experiments have revealed two aspects, or levels, of competition between motoneurons. In the urodele limb the competition leads to restoration of proper co-ordination, largely by motor nerves regaining control of the muscles for which their central connections were most appropriate. This most likely reflects a peak affinity between each motor nerve and its appropriate muscle, and vice-versa. In mammals, where the equally vigorous regeneration of motor nerves does not restore proper co-ordination (review by Mark, 1969), a second aspect of competition is exposed. Competition between muscle fibres for terminals, by terminal sprouting, leads to saturation of the postsynaptic fields, and conversely, competition between motoneurons for terminals, in a manner related to the amounts of transmitter released, limits the number of terminals made by each neuron.

The second aspect of competition, that of saturation, is essential to the former, since competition between neurons of graded affinity would otherwise lead to the neurons with the highest affinity occupying *all* the postsynaptic sites (Prestige and Willshaw, 1975).

The signals for sprouting are unclear (reviewed by Brown, Holland and Hopkins, 1981). Nodal sprouting appears to involve Schwann cells (Hopkins and Slack, 1981) whereas terminal sprouting may involve a diffusible agent. Nerve death results in movement of Schwann cells into the space previously occupied by the nerve terminal, where they effect spontaneous miniature end-plate potentials in the postsynaptic cells (Kriebel, Hanna and Pappas, 1980). Similarly within the central nervous system, microglia are closely associated with the loss of synaptic boutons on axotomised motoneurons (Grafstein, 1975). Although the initial sprouting reaction appears to be a local one, as it may occur in motor axons separated from the cell body (Rotshenker, 1981), it ultimately affects the soma (McIlwain and Farel, 1979), and other motoneurons in some cases (Rotshenker, 1979).

Conditions may differ in development, in that Schwann cells are not present during the growth of motor axons into the frog's limb, but later migrate down alongside the axons (Prestige and Wilson, 1980). The substantial loss of motoneurons that have axons in the periphery at these early stages suggests that motoneurons may have a limited sprouting capacity at this time, in contrast to the situation in the adult (Prestige and Wilson, 1974).

1.12 ELIMINATION OF POLYNEURONAL INNERVATION IN MAMMALS

The extrafusal muscle fibres of adult mammals are generally innervated by one neuromuscular junction equidistant from the ends (Bagust, Lewis, Pallot and Westerman, 1972). Each alpha motoneuron may supply several hundred fibres, generally within the same muscle (see Emonet-Dénand, Laporte and Proske, 1971 for an exception). The fibres may be divided into two main categories, fast twitch and slow twitch, on the basis of their speed of contraction and resistance to fatigue (Denny-Brown, 1929; Stein and Padykula, 1962; Guth, Samaha and Albers, 1970; Burke, Levine, Tsairis and Zajac, 1973; Burke, Levine, Salcman and Tsairis, 1974). The fast twitch fibres may be further divided according to contraction and histochemical properties (Close, 1967). Fibres of both types may occur in a single muscle (Burke, Levine, Tsairis and Zajac, 1973) and the percentage of each type is characteristic of the muscle (Burke, 1967; Burke, Levine, Tsairis and Zajac, 1973).

The fibre properties are determined predominantly by the motoneuron. Every fibre contacted by a particular motoneuron has identical histochemical properties (Edstrom and Kugelberg, 1968; Burke, Levine, Tsaisis and Zajac, 1973). Cross-innervation of slow muscle by a predominantly fast motor nerve, and vice versa, results in at least a partial transformation of the fibres (Buller, Eccles and Eccles, 1960 a, b; Close, 1965, 1969). Motoneurons innervating slow twitch fibres have smaller somas than those innervating the larger fast twitch motor units (Granit, Phillips, Skoglund and Steg, 1957; Eccles, Eccles and Lundberg, 1958; Henneman, Somjen and Carpenter, 1965).

Skeletal muscle fibres in new born mammals are all slow. Fast fibres arise postnatally, between weeks 3 and 6 in the kitten (Denny-Brown, 1929; Buller, Eccles and Eccles, 1960 a) and weeks 1 to 5 in the rat (Close, 1967). In kittens this is matched by the differentiation of alpha motoneuron size classes within the spinal cord (Sato, Mizuno and Konishi, 1977).

In developing muscle, 'primary' myotubes form by fusion of mononucleate myoblasts, prior to innervation (Kelly and Zacks, 1969). Subsequent proliferation requires functional innervation (Engel and Karpati, 1968; Betz, Caldwell and Ribchester, 1980; Harris, 1981). 'Secondary' myotubes arise near the centre of primary myotubes (Ontell, 1977) and may rapidly outnumber the primary fibres (Kelly and Rubinstein, 1980). In rat gastrocnemius these secondary fibres are located in the superficial region of the muscle (Rowlerson, 1980). In rat diaphragm, secondary fibres are generated throughout the muscle (Harris, 1981). The adult total of fibres is attained in rat lumbrical muscle provided at least eight of the normal 11 or 12 motor units are present at birth (Betz, Caldwell and Ribchester, 1980). This suggests that although innervation is necessary, it does not directly regulate fibre proliferation.

At birth each muscle fibre is found to receive polyneuronal innervation, from 4-5 axons terminating at a single end plate. The number is subsequently reduced to the adult pattern of only one axon per terminal, over several ensuing weeks (Redfern, 1970;

Bagust, Lewis and Westerman, 1973; Betz, Caldwell and Ribchester, 1979, 1980; Dennis, Ziskind-Conhaim and Harris, 1981). The loss is dependent on functional innervation (Benoit and Changeux, 1975; Brown, Jansen and Van Essen, 1976), and is unaffected by deafferentation (Caldwell and Ridge, 1981).

Despite the increase in numbers of muscle fibres postnatally there is a reduction in motor unit size (Brown, Jansen and Van Essen, 1976; Betz, Caldwell and Ribchester, 1980). In rat soleus muscle individual motor units are reduced from occupying 23%, to 4-5% of the muscle over this period (Jansen, Van Essen and Brown, 1975).

Brown, Jansen and Van Essen (1976) reported that the total number of motor units did not diminish during loss of polyneuronal innervation in rat muscle, implying that it was not associated with neuron death. Hutchinson, Davey and Bennett (1981) found a loss of axons in the eighth cervical ventral root during loss of polyneuronal innervation from the biceps muscle. It has been suggested that motoneuron death in the brachial spinal cord of the neonatal rat may be associated with this loss of innervation (Nurcombe, McGrath and Bennett, 1981). A similar loss of polyneuronal innervation occurs in the chick forelimb between days 9 and 20 of incubation and possibly earlier (Pettigrew, Lindeman and Bennett, 1979) and motoneuron numbers gradually decline from 17000 at day 6 to about 7000 at day 21 in the chick brachial motor column (Oppenheim and Majors-Williard, 1978).

In the rat the dorsal root connections onto the motoneurons are formed prior to birth. During the first three weeks after birth the limb motoneurons receive long descending segmental and supraspinal inputs (Gilbert and Stelzner, 1979). Although these events in some cases coincide with the period of brachial motoneuron death, there is no evidence to suggest that they are a contributing cause.

1.12.1 Development of Muscle Spindles

Motoneurons which innervate muscle fibres within muscle spindles (intrafusal fibres) are termed gamma motoneurons, those innervating extrafusal fibres exclusively are termed alpha motoneurons (reviewed by Matthews, 1972) and those innervating both intrafusal and extrafusal fibres are beta motoneurons (Emonet-Dénand, Jami and Laporte, 1975).

Gamma motoneurons are distributed among the alpha motoneurons to the same muscle, as judged electrophysiologically, and morphometrically (Eccles, Eccles, Iggo and Lundberg, 1960; Nyberg-Hansen, 1965; Bryan, Trevino and Willis, 1972; Burke, Strick, Kanda Kim and Walmsley, 1977) and comprise 10-23% of the mouse hindlimb motor pools (McHanwell and Biscoe, 1981).

Mammalian muscle spindles generally receive only one primary afferent termination (reviewed by Barker, 1974). A single gamma fibre usually supplies several spindles within a muscle (Brown and Butler, 1973). Gamma motoneurons differ histochemically from

alpha motoneurons. Their low phosphorylase and high succinic dehydrogenase activities are the reverse of those of the alpha motoneurons, but are similar to those of spinal interneurons (Campa and Engel, 1970, 1971). They also differ in that they project to muscle fibres that have a wide range of structural and histochemical properties (Brown and Butler, 1973), and do not receive monosynaptic projections from group Ia afferents (Eccles, Eccles, Iggo and Lundberg, 1960; Grillner, Hongo and Lund, 1969).

Muscle spindles in rat limbs usually contain 2 bag and 2 chain fibres. Those in cats usually contain 2 and 4 respectively (Banker and Girvin, 1971). In the rat these fibre differences arise postnatally. Spindles can be first detected at about 19 days in the rat foetus (gestation period 21 days) and consist of a single bag fibre and sensory terminal (Barker and Milburn, 1972). By the 4th postnatal day the full complement of four intrafusal fibres is present, together with recognisable motor terminals. The intrafusal fibres differentiate over the next 8 days (Milburn, 1973). The spindles do not form if the muscle is denervated prior to birth (Zelená, 1957). Denervation at birth results in their degeneration within 10 days, but has no detectable effect at 14 days (Zelená and Hnik, 1963). The size distribution of motoneurons in the kitten spinal cord is already bimodal at birth, suggesting that the alpha and gamma motoneurons have begun to differentiate at that stage (Sato, Mizuno and Konishi, 1977).

1.13 SUMMARY

The following tentative scheme can be drawn:

1. The motor, but not sensory centres of the spinal cord are embryologically determined prior to axon outgrowth (Section 1.4), by means that are largely unknown (Section 1.5).
2. The motor axons follow the same course as the axons innervating the myotomes, to reach the base of the limb (Section 1.8).
3. The axons enter the limb bud when its mesenchyme is mostly undifferentiated. Morphogenetic forces within the limb bundle the axons into the main nerve trunks, and terminals are formed. The sensory axons follow the motor axons into the limb, and are, at least partially, specified by the variety of receptors they induce (Section 1.4).
4. There is some topographic matching of motoneurons in the cord, with their muscles in the limb. Variability in the relative positions and growth of the cord, intervening myotomes, and limb, and other factors render this match imprecise. Some neuron connections are then less appropriate than others.
5. The maturing peripheral neurons become dependent on their peripheral connections. Competition between neurons then eliminates those with the least appropriate peripheral and central connections. In this way the nervous system is reproduced within the bounds of the inheritance, but without the necessity for a strictly pre-determined plan.

SUMMARY OF EXPERIMENTAL MATERIAL

Two main groups of experimental animals were used. The first group of 10 was used for Chapters 2, 3 and part of 4, the second group of 20 was used for Chapter 4. Due to the loss, reduplication, resorbtion etc. of transplants, over 200 operations were performed to obtain these groups.

CHAPTER 2

Composition of the lumbar plexus - 22 controls (unoperated)
10 experimental (A,B,C,D,E,F,H,I,J,K)

Muscle and nerve pattern of sectioned limbs - 4 controls (unoperated)
6 experimental (A,C,D,H,I,J)

CHAPTER 3

Neurons on the unoperated side of the spinal cord were used as controls

Motoneuron counts - 10 experimental (A - K)

DRG neuron counts - 6 experimental (A,B,C,H,I,J)

Motoneuron nuclear areas - 7 experimental (A,B,C,D,E,F,J)

Myotome transplants - 4 experimental (M,N,O,P) - 78 operations

CHAPTER 4

Motor and reflex behaviour - >20 controls (unoperated)
- experimental (A - K)

Motor somatotopy - 20 experimental, 11 failures due to poor injection or HRP label diffuse or absent in motoneuron somas.

CHAPTER TWO

THE PROJECTION FROM THE LUMBAR SPINAL CORD TO A TRANSPLANTED FORELIMB IN *XENOPUS*

I. SEGMENTAL SUPPLY AND BRANCHING PATTERN OF THE LIMB NERVES

2.1 INTRODUCTION

A fundamental problem in development of the nervous system is the situation where one set of neurons must connect in an orderly manner with another set at a remote location. The problem is simplified if any topographic order that may exist between neurons of the presynaptic set is also matched by the postsynaptic set, for then the requirements of axons to re-order during their passage is diminished.

The topographic order of muscles in the vertebrate limb is matched to some extent by the topography of their motoneuron pools within the spinal cord (Rexed, 1952; Romanes, 1964; Székely and Czéh, 1967; Cruce, 1974 a; Lamb, 1976; Landmesser, 1978 a; Hollyday, 1980 b; McHanwell and Biscoe, 1981 a; Frank and Westerfield, 1982 a). Proper co-ordination of the limb muscles is eventually restored following surgical disorganization of the limb nerves in urodele amphibia (Grimm, 1971; Cass, Sutton and Mark, 1973). This recovery is characterized by the regeneration of motor nerves to their original muscles, without restoration of the original pattern of branching of the limb nerves (Grimm, 1971; Cass, Sutton and Mark, 1973; Bennett and Raftos, 1977), and depends at least partly on competition at the neuromuscular junctions (Yip and Dennis, 1976; Bennett and Raftos, 1977; Dennis and Yip, 1978; Bennett, McGrath and Davey, 1979; Wigston, 1979).

Similarly, lower vertebrates may regain orderly visual functions following disruption of the optic axons leading from the retina to the tectum (Sperry, 1943 a, 1945 b). This led to the proposal that the selectivity of neuronal connections must be based on cytochemical matching of neurons (Sperry, 1963) in addition to any topographic matching that may exist between each set.

It is not yet clear how motoneurons connect to the muscles for which their central connections are most appropriate, during development. Topographic order and a temporal sequence of outgrowth of motor axons might achieve this correspondence (Horder, 1978). Additional selective processes must occur however, since the number and origin of segmental nerves to the limb varies between individuals of the same species (Sherrington, 1892; Cruce, 1974 a), and where several segments of the spinal cord are translocated or reversed, motoneurons may still connect with their appropriate muscles (Lance-Jones and Landmesser, 1980, 1981 b).

Selectivity might occur by the growth of axons from prespecified motoneurons, to the appropriate muscles (Lance-Jones and Landmesser, 1981 a, b), or alternatively, the axons might grow and terminate unselectively, with the least appropriate, in terms of their central connections, being eliminated by the death of some neurons (Hughes, 1968; Lamb, 1977; Pettigrew, Lindeman and Bennett, 1979; Mark, 1980).

There have been a number of investigations of the selectivity of growth, or otherwise, of axons in foreign tissues (Wenger, 1950), but only a few have examined the branching pattern of limb nerves

in the heteronymous limb. Braus (1904, 1905) transplanted forelimb buds to the head or trunk of *Bombinator* and stated that the branching pattern resembled that of a normal limb. Hamburger (1939 b) reported a normal plexus and sciatic nerve in a hindlimb supplied by brachial segments in *Gallus*. Piatt, however, reported abnormalities in the pattern of nerves supplying aneurogenic (1952) or rudimentary (1956) forelimbs transplanted in place of the hindlimb of *Amblystoma*.

In this study a hindlimb bud of the *Xenopus* tadpole was replaced prior to axon invasion with that of a forelimb. The segmental composition of the nerve plexus, and the branching pattern of the limb nerves were examined after metamorphosis.

2.2 METHODS

Embryology: Batches of *Xenopus laevis* eggs were obtained following injection of adults (Xenopus Limited, U.K.) with human chorionic gonadotropin (Sigma, St. Louis). Tadpoles were raised in dechlorinated, aerated tapwater at 17-22°C and fed on nettle tea powder, or liver pieces after metamorphosis. Embryological operations incorporating several batches were performed in *Holtfreter* solution, of composition 60mM NaCl, 0.67mM KCl, 0.80mM $CaCl_2$ and 0.24mM $NaHCO_3$ (Rugh, 1962). Each animal rested in a Sylgard-lined (Dow-Corning, Midland) dish with illumination from below by a fibre optic light source (Volpi, Zurich) and was viewed at magnifications of 6 to 50X with a Wild M5 microscope (Heerbrugg, Switzerland). MS222 (ethyl-m-aminobenzoate, Sigma, St. Louis) was added to the solution as required to sustain a sufficient level of anaesthesia. With

iridectomy scissors the right forelimb bud of a stage 50 (Nieuwkoop and Faber, 1967) tadpole was amputated at its base. The right hindlimb bud of a stage 49 host was then amputated, as much mesenchyme as possible scraped from the site, and the forelimb bud transplanted in its place using tungsten needles. Forelimb epidermis was spread over as much of the wound as possible to prevent hindlimb regeneration (Tschumi, 1957). Recovery took place over 24 hours in Holtfreter solution. Most operations were performed during the summer period January - March.

Segmental innervation: The segmental nerve supply to the limbs was determined in 10 operated and 22 normal frogs, after metamorphosis. Normal frogs were within the size range of the operated group. The number and relative diameter of the nerves was recorded with the aid of a Zeiss binocular microscope at magnifications of 5 to 50 X.

Histology: The pattern of branching of nerves was determined from serial sections of 6 operated and 4 normal limbs, and confirmed in other specimens by dissection. Limbs were serially sectioned at 20μm thickness, and silver-impregnated using Palmgren's technique (Palmgren, 1948). Even with young animals, the amount of myelin surrounding the nerve axons made double impregnation with silver necessary. Sections were drawn with the aid of a camera lucida, at intervals of 200μm, and the pattern of nerve trunks reconstructed from these drawings.

Photography: Colour prints were made from slides using Agfa CT21 daylight film (A.S.A. 100), taken with a Nikon camera and attached Micro-Nikkor 55 mm lens.

TABLE 2.1a NORMAL HINDLIMB VS TRANSPLANT FORELIMB WEIGHTS

(Weights in milligrams, following fixation)

NORMAL HINDLIMB	TRANSPLANT FORELIMB	RATIO (%) FORELIMB / HINDLIMB
947	142	15
653	111	17
71	10	14
153	23	15
215	28	13
4517	542	12
8073	1211	15
5095	1019	20
4124	701	17
5031	805	16

Black and white prints were made from Kodak Pan X negatives (A.S.A. 32), using either a Nikon Fl camera, or Wild Photoautomat MPS 55 attached to a Leitz Ortholux II microscope, or an M400 Photomakroskop.

2.3 RESULTS

Development of Transplanted Limb Buds: At the time of amputation the hindlimb bud was circular, 0.2 mm in diameter, and its mesenchyme was undifferentiated. Motor axons can be first detected in the bud at stage 50, by histochemical labelling of motoneuron somas after injection of horseradish peroxidase into the bud (Lamb, 1974). Lateral column motoneurons are produced until stages 52-53 (Prestige, 1973) therefore few, if any axons would have entered the hindlimb bud prior to its removal. Some motoneurons can regenerate their axons at these stages (Lamb, 1981 c). They are not sensitive to axotomy until stage 53 (Prestige, 1967 a), by which time the transplant limbs were well established and had undergone considerable growth and differentiation.

The transplanted forelimb bud was slightly larger than the stage 49 member, and may have received its first motor axons from brachial neurons (Nieuwkoop and Faber, 1967). Successfully transplanted buds acquired a blood supply within 24 hours. About 10% underwent self-differentiation (Braus, 1904, 1905) in the hindlimb position (Fig. 2.1 (a), (c)), to form a morphologically normal forelimb at juvenile stages (Fig. 2.2 (a), (b), (c), (d)). None of the limbs were conspicuously hypertrophic.* A left or right limb arose

*see Table opposite.

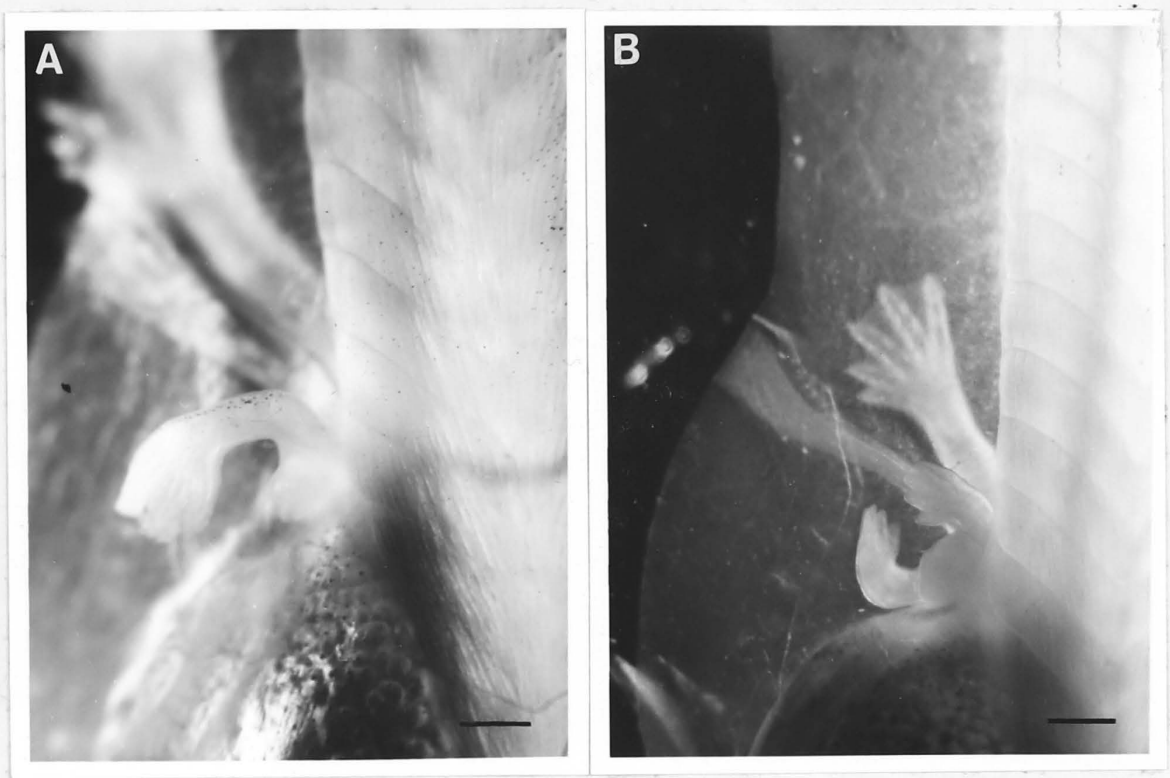
FIGURE 2.1 DEVELOPMENT OF TRANSPLANTED FORELIMBS IN THE HINDLIMB POSITION IN *XENOPUS LAEVIS*

(a) Transplanted forelimb at stage 58

(b) Duplicated forelimb. The limbs have mirror-image symmetry

(c) Transplanted forelimb at stage 58

Bars = 1mm.


A
B


C

FIGURE 2.2 *XENOPUS LAEVIS* TOADS WITH A TRANSPLANTED FORELIMB IN THE HINDLIMB POSITION

(a) Animal J

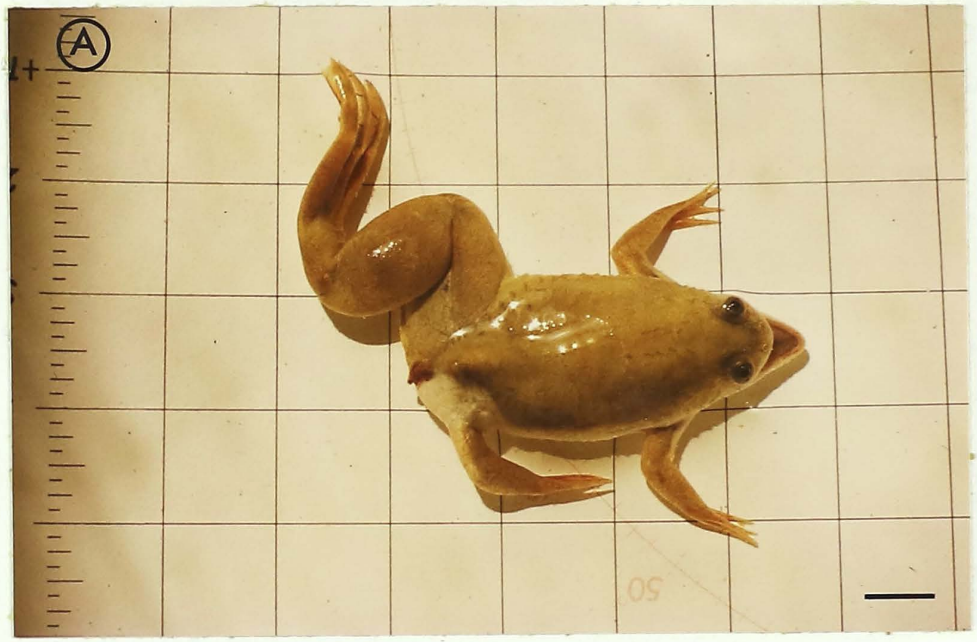
(b) Animal J

(c) Animal K

(d) Animal K

The limbs show only slight hypertrophy

Bars = 1cm.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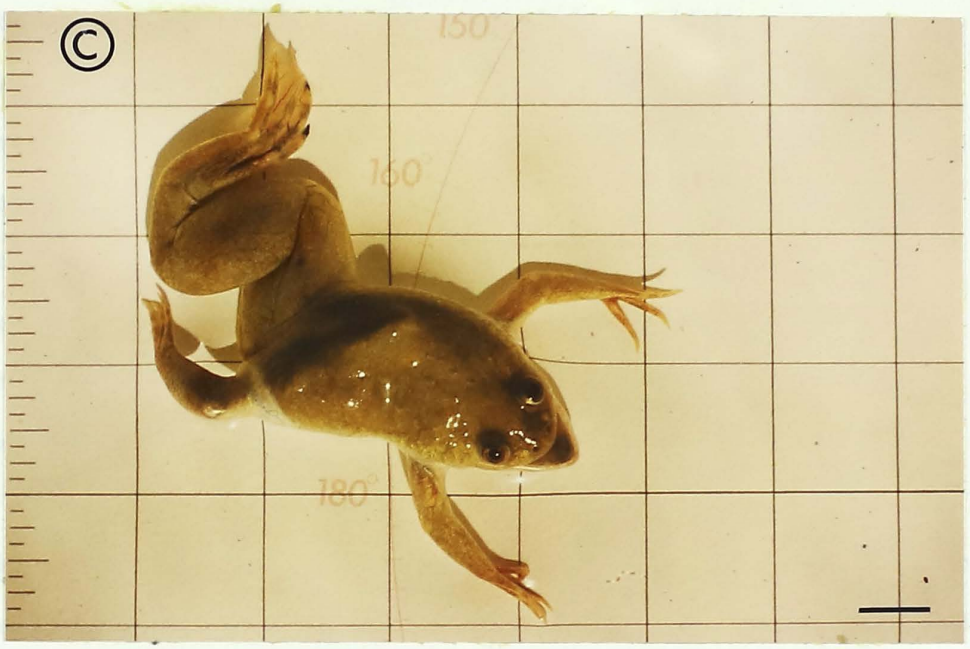
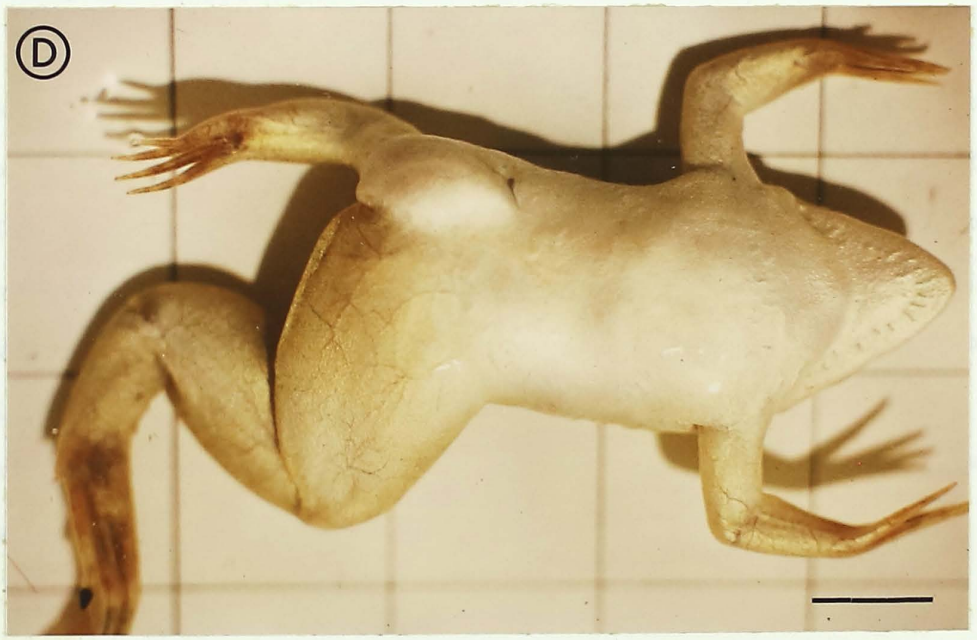
B


C
150
160
180


D

according to its original donor. Well-formed limbs contained a shoulder joint, and some bones of the pectoral girdle onto which transplant muscles made their usual insertions. The most complete limb incorporated a scapula, suprascapula, coracoid and clavicle. Each limb was loosely held in position by its skin, and physical constraints on the shoulder and girdle components, without fusion of muscle or bone to the contralateral pelvic bones (Fig. 2.3 (a), (b), (c), (d), (e), (f), (g)).

Abnormalities of Limb Development: Careful attention was given to any abnormalities of the limbs (Fig 2.1 (b), 2.4 (a), (b), (c), (d)). Hindlimb regeneration almost always occurred after loss of a transplant at stages 49-50, (Tschumi, 1957), and frequently when the differentiated epidermis of the transplant did not completely cover the hindlimb site (Fig. 2.4 (a)). The regenerated hindlimb and transplant forelimb were both mobile and sensitive.

Another common abnormality was the duplication of the distal structures of the limb (Harrison, 1921; Maden, 1981; Muneoka and Bryant, 1982). The duplication always arose at the level of the pectoral bones, or proximal humerus. At early stages each member of a duplicate pair was smaller than the normal forelimb, but after metamorphosis each limb had reached almost normal size (Fig. 2.4 (b)). A stage 50 hindlimb bud in place of the forelimb may also develop supernumerary structures (unpublished observations).

FIGURE 2.3 PELVIC GIRDLES OF OPERATED ANIMALS

Pelvic bones and musculature are absent on the operated side

(a) Ventral view of Animal K, bar = 5mm.

(b) Ventral view of Animal K

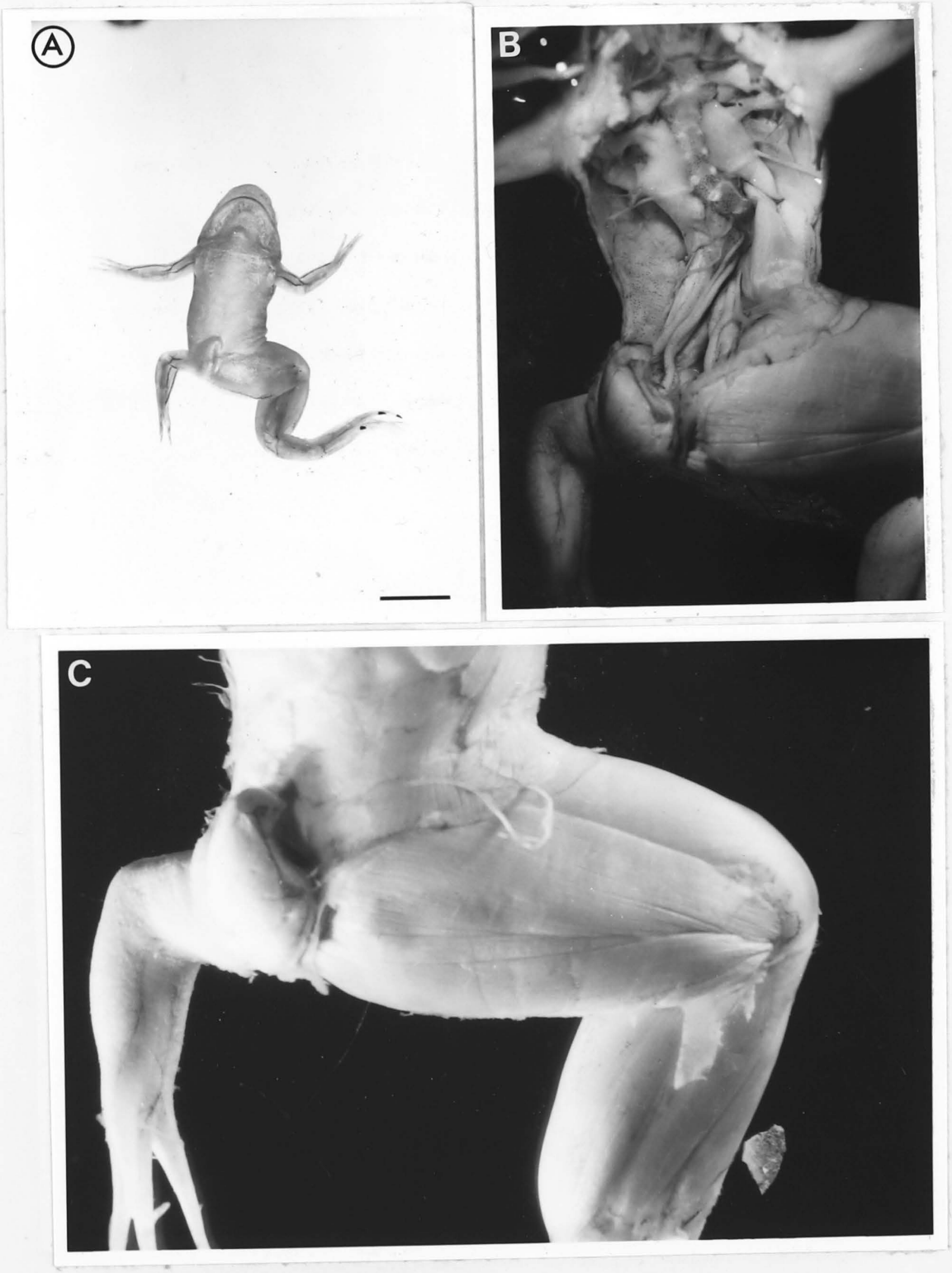
(c) Ventral view of Animal K

(d) Dorsal view of Animal J, bar = 1mm.

(e) Ventral view of Animal J

(f) Ventral view of Animal A, viscera removed, bar = 1mm.

(g) Ventral view of Animal B, viscera removed, bar = 1mm.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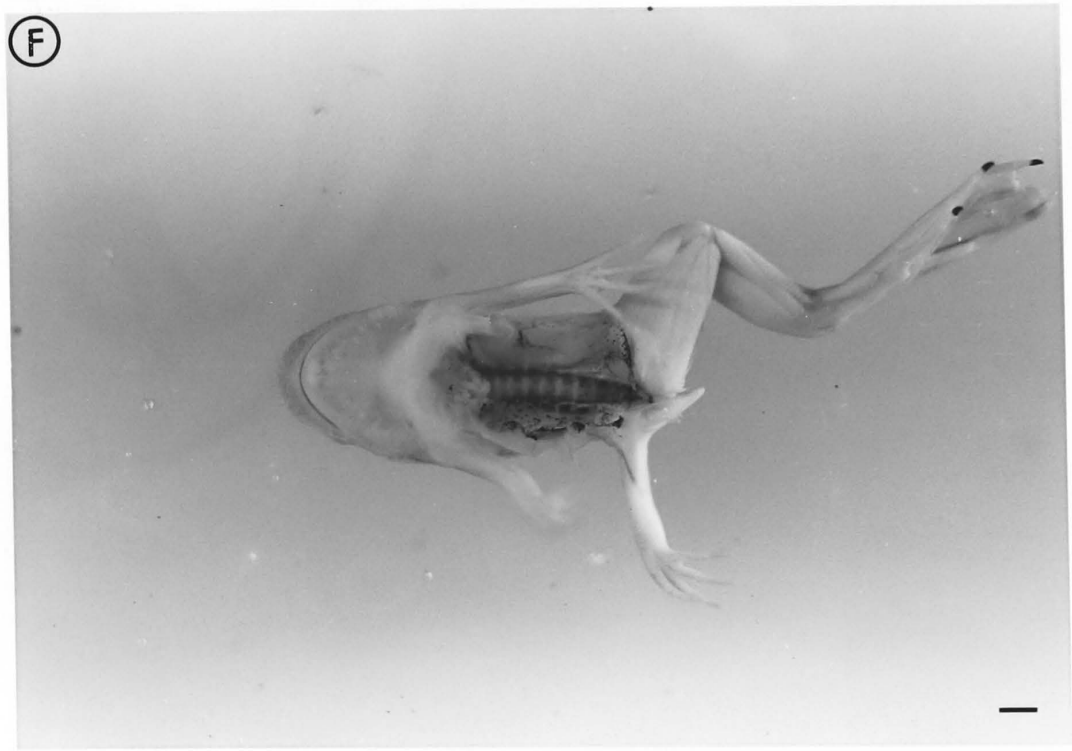
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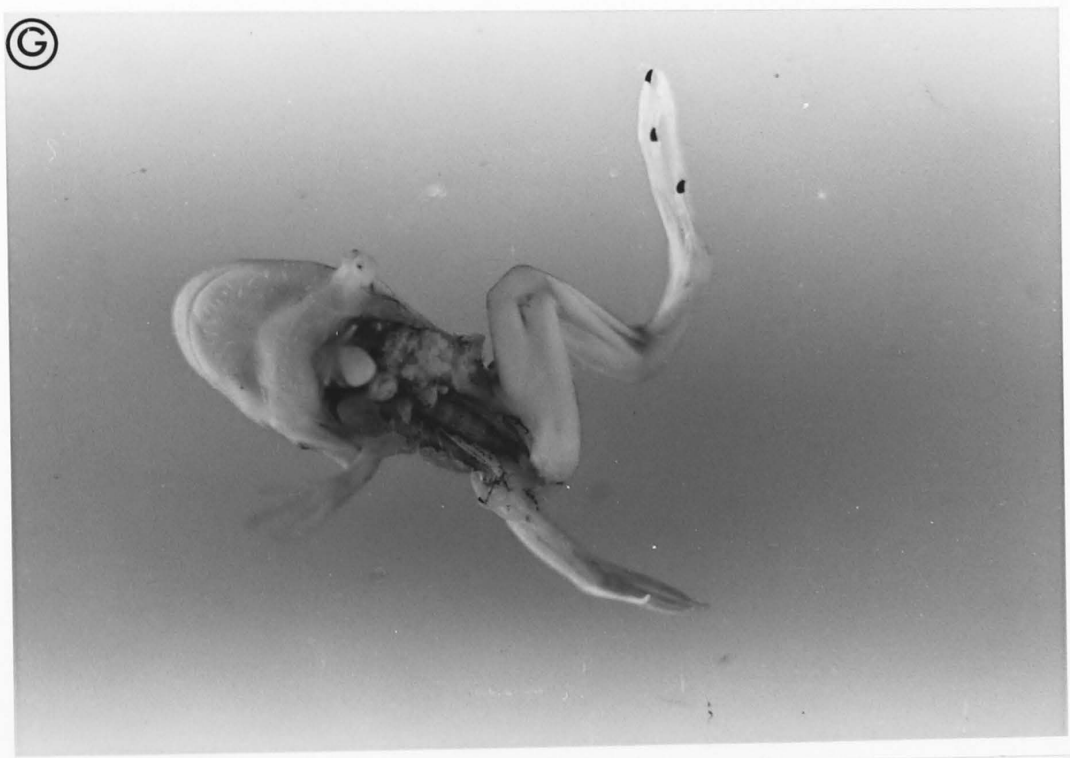
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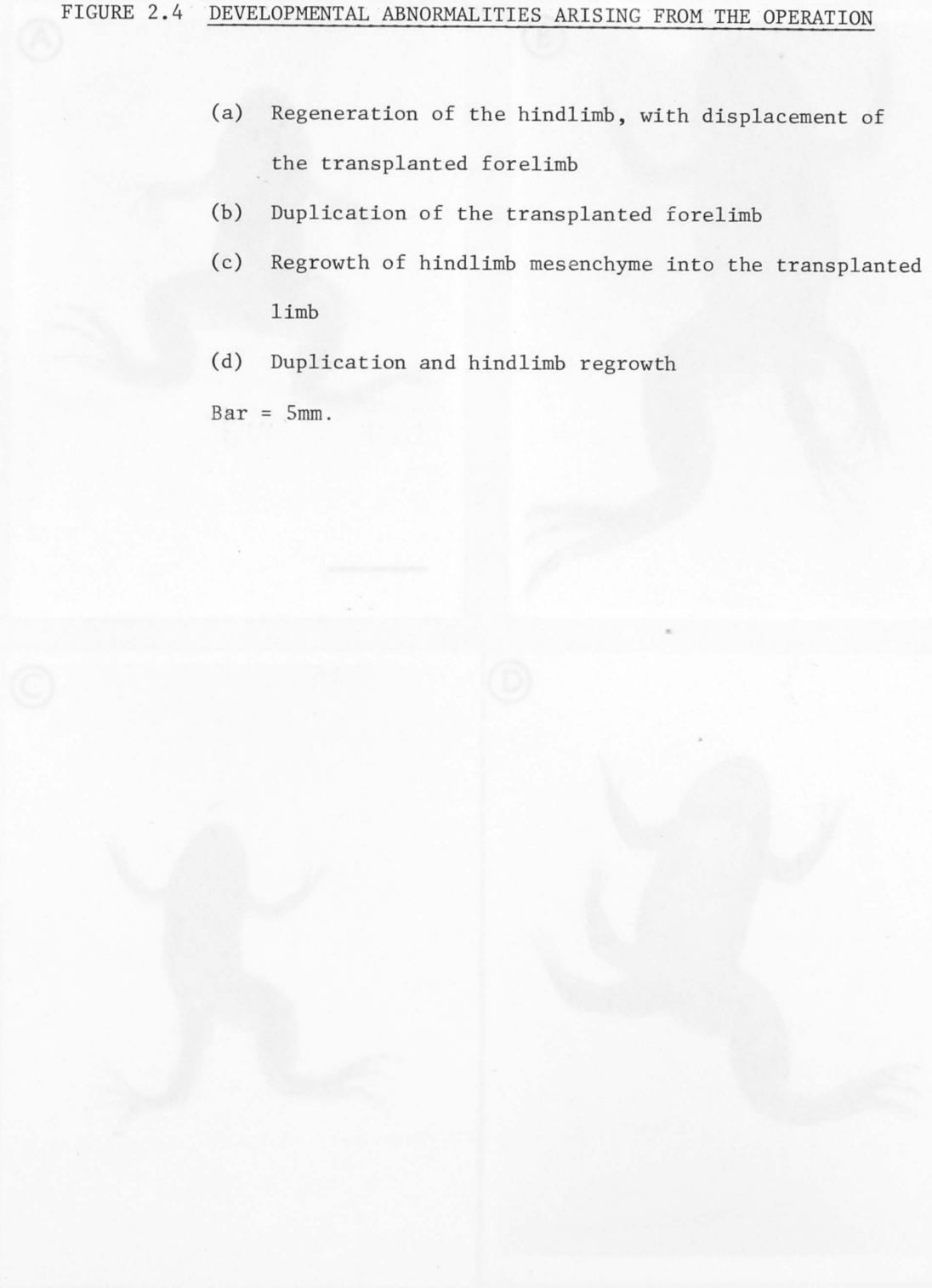
FIGURE 2.4 DEVELOPMENTAL ABNORMALITIES ARISING FROM THE OPERATION



(a) Regeneration of the hindlimb, with displacement of the transplanted forelimb

(b) Duplication of the transplanted forelimb

(c) Regrowth of hindlimb mesenchyme into the transplanted limb

(d) Duplication and hindlimb regrowth

Bar = 5mm.


A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D

Less frequently some transplanted forelimbs also contained thigh musculature; and in several cases an extra digit, or some claws were present (Fig. 2.4 (c), (d)). This was most likely due to hindlimb mesenchyme invading the transplant. The rapid proliferation of hindlimb mesenchyme made this form of regeneration conspicuous in the comparatively small forelimb, especially prior to metamorphosis when the skin of the limb was translucent and the underlying musculature readily visible.

TABLE 2.1 SEGMENTAL COMPOSITION OF THE NERVE PLEXUS OF NORMAL AND TRANSPLANTED LIMBS OF *XENOPUS*

	Number and Relative Diameters of Nerves (largest diameters underlined)	
Normal Forelimb	2<<3 = 4	2
	3	1
	3 = 4	41
		44
Normal Hindlimb	8 = 9>10	2
	8<9>10	8
	8<9 = 10	2
	8<9<10	7
	8<9<10>>11	3
	8<9 = 10>>11	5
	8<9<10>>11	15
	8<9<10>11	2
		44
Forelimb Transplanted in Place of Hindlimb	8 9>10	3
	8<9>10	4
	8<9 = 10	1
	8<9<10	2
		10

Segmental Composition of the Nerve Plexus of Normal and Transplanted Limbs: (Table 2.1 Fig. 2.5)

The convention of Gaupp (1896) was adopted, in which nerve 1 was lost during metamorphosis, leaving nerves 2 and onwards. In 22 normal animals the forelimb plexus was most commonly supplied by segmental nerves 3 and 4. In an exceptional case nerve 3 was the sole contributor, and was twice normal size, with nerve 4 being unusually small. The supply to the contralateral forelimb in this case was normal. The hindlimb plexus was most commonly supplied by nerves 8, 9, 10 and 11, sometimes 11 was absent.

All 10 transplanted forelimbs were supplied by lumbar nerves 8, 9 and 10 (Figs. 2.5 (b), (c)) and these nerves were of smaller diameter than the corresponding contralateral set. Nerve 11 was not seen to contribute to the limb. It may have been too small to be visible, or it may have taken a different route to a non-limb target.

Entry of Nerves to the Transplanted Limb:

In 8 of the 10 limbs the 3 lumbar nerves united, and after giving small branches to muscles of the pectoral girdle, they entered the limb in a single trunk (Fig. 2.6 (a), (b)), containing both sensory and motor axons. In the other 2 limbs only 2 of the 3 nerves united prior to entering the limb (Fig. 2.6 (c), (d)), although these limbs had a more complete pectoral girdle than most of the others. Irrespective of the orientation of the limb, all the lumbar nerves entered the limb at the same point as in a normal forelimb, passing

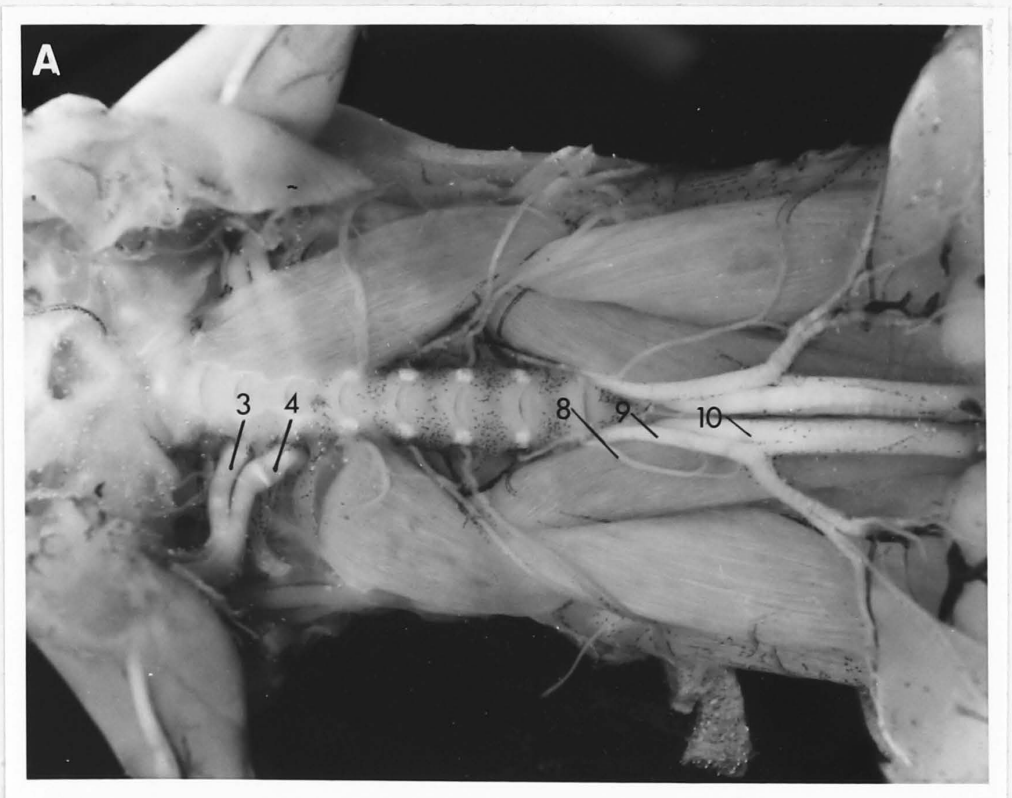
FIGURE 2.5 SEGMENTAL SUPPLY TO NORMAL AND TRANSPLANTED LIMBS

(a) Normal supply. The brachial nerves to the right forelimb have been exposed

(b) Segmental nerve supply to a transplanted forelimb on the right-hand-side of the animal. The spinal column has twisted due to the lack of pelvic bones and musculature on one side

(c) Segmental nerve supply to the transplanted forelimb of Animal B

Bars = 1mm.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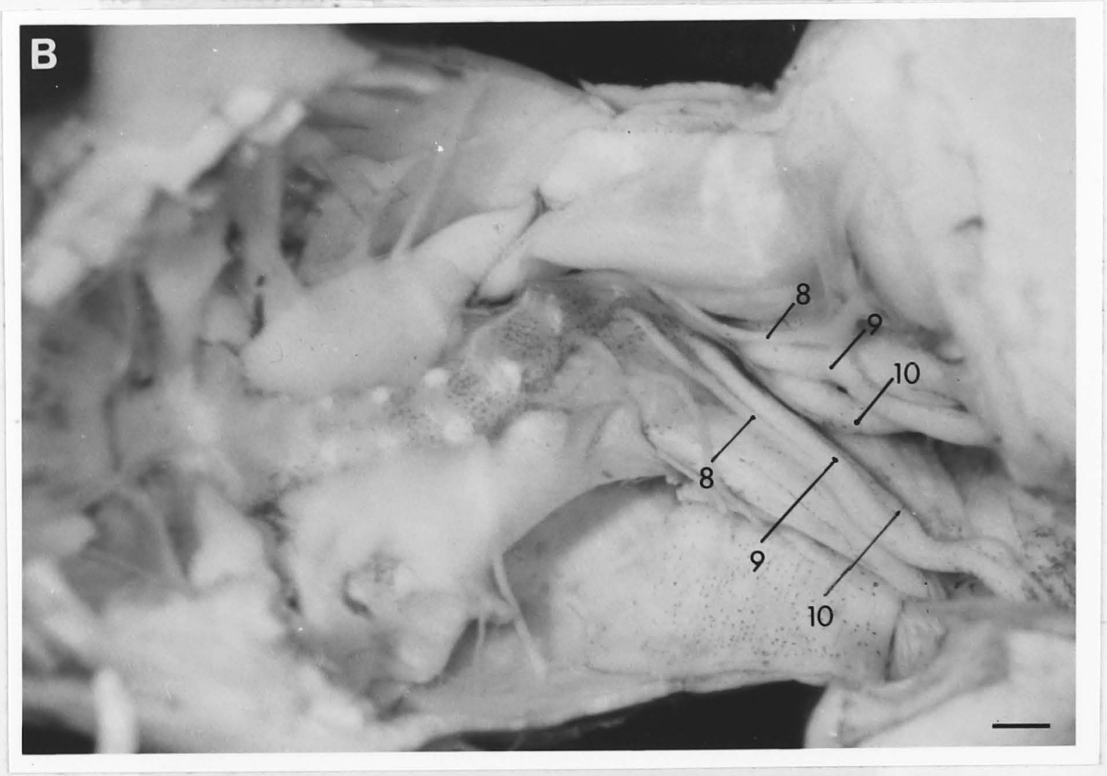
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between heads of the M. triceps (Fig. 2.6 (d)), on the side appropriate to the handedness of the limb.

Branching Pattern of the Limb Nerves:

Before fixation, all the limbs examined had complete flexibility of the joints, including the shoulder, and in cross-section the bones, joints, tendons and ligaments were found to have formed properly. For example, the long ligament running parallel to the humerus, by which means the M. coraco-radialis inserted onto the radioulna, was always present (Fig. 2.7 (a)). It was also apparent, even with the non-specific histological processing involved, that there was regional differentiation of the fibres of each muscle (Fig.2.7 (a), (b)). It is not known whether this corresponded to the particular categories of fast and slow fibre type found in amphibian limb muscles (Luff and Proske, 1976).

The major nerve branches of the normal forelimb could be recognized in transplanted forelimbs. Shortly after entry to the limb, the single nerve trunk divided, with one branch passing between the M. triceps and humerus to the outer surface of the upper arm, as the N. radialis, while the other branch constituted the N. ulnaris (Fig. 2.7 (a)). At the point of division 3 branches supplied the M. triceps externus, internus, and medialis. At the mid-level of the humerus the cutaneous branch of the N. ulnaris (Fig. 2.7 (a), (b)) ran to the skin of the inner surface of the forearm and hand. At a more distal level, the cutaneous branch of the N. radialis ran to the outer skin of the forearm and hand. Within the forearm the radial and ulnar nerves gave off several

FIGURE 2.6 ENTRY OF LUMBAR NERVES TO TRANSPLANTED FORELIMBS

(a) Animal I

(b) Animal K

(c) Animal H

(d) Animal H

Bars = 1mm.


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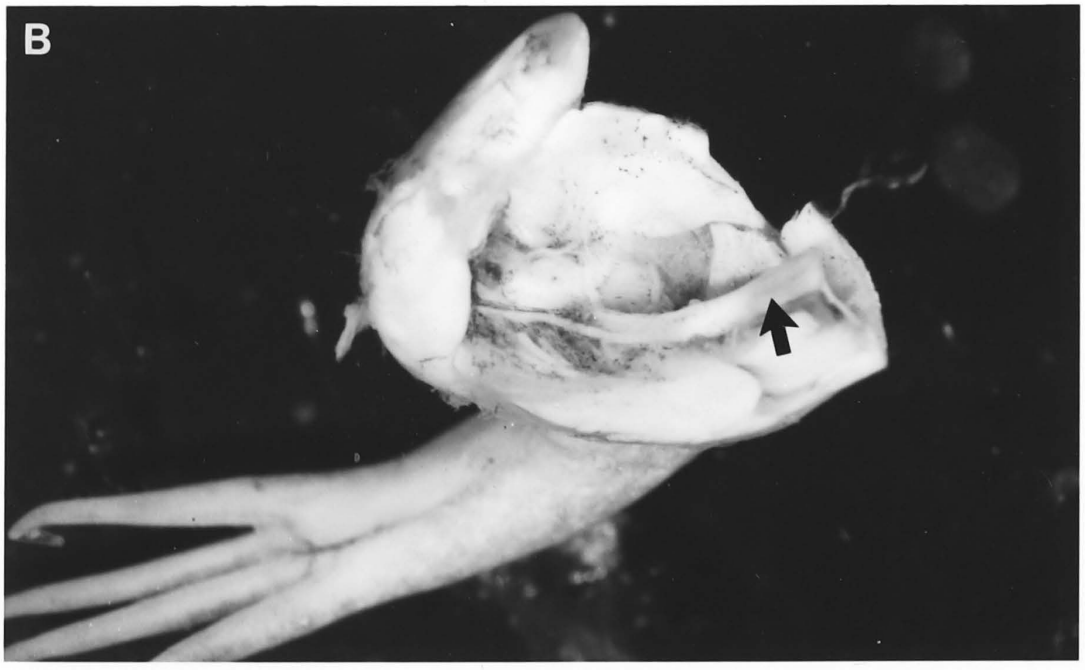
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FIG. 2.7 TRANSVERSE SECTIONS OF TRANSPLANT AND CONTROL FORELIMBS

The sections are approximately equally spaced along the proximodistal axis, from the humerus to the carpals. The upper plate on each page is the transplant limb, the lower plate is a normal control limb at the same axis level. The nerves are shown by blue arrows, the muscles, bones and tendon are shown by red arrows. The nerve branches in F and G are too small to be seen clearly and are not marked.

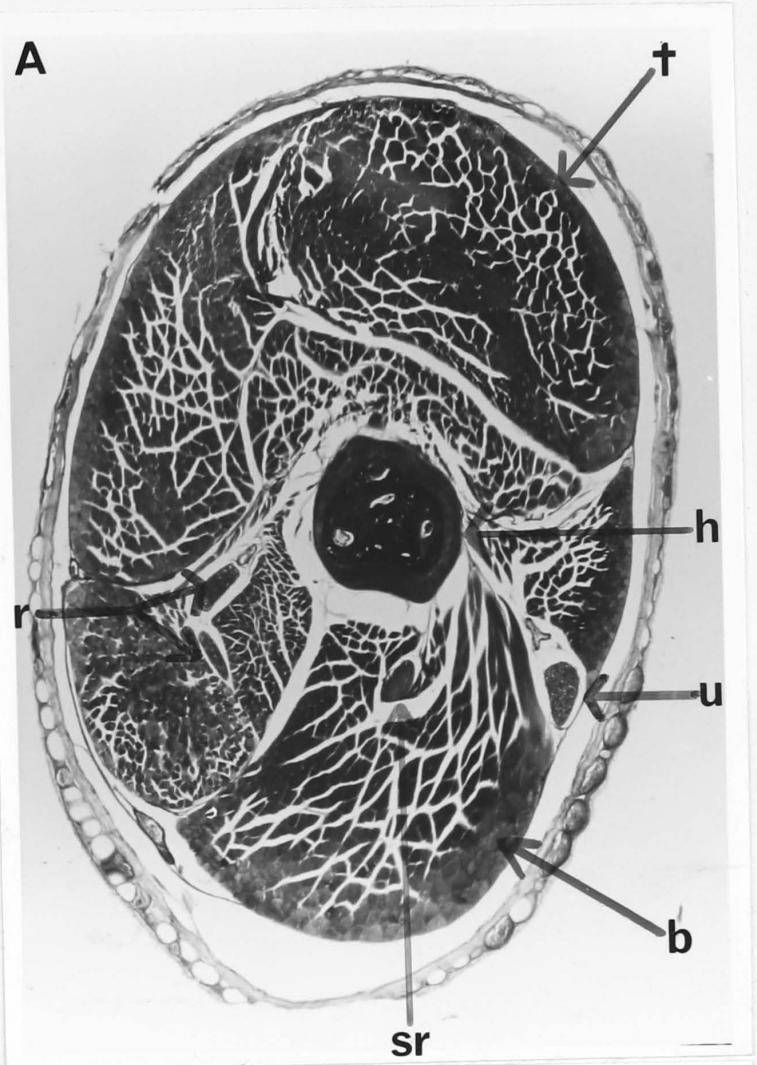
Note the close correspondence between the nerve pattern in the experimental and control limbs. The nerves in the transplant limb occupy almost identical positions in relation to the surrounding muscles, blood vessels and bones.

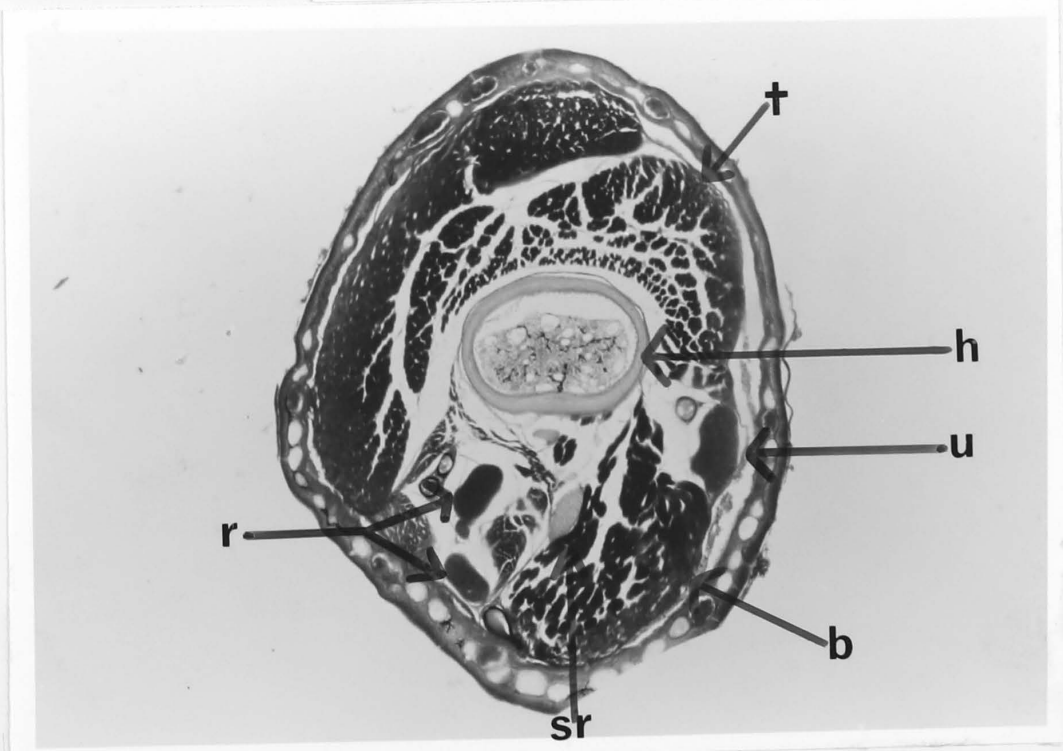
The nerves in the transplant limb are of smaller relative diameter than in the control, most likely due to the smaller number of supply neurons in the central nervous system (see Chapter 2).

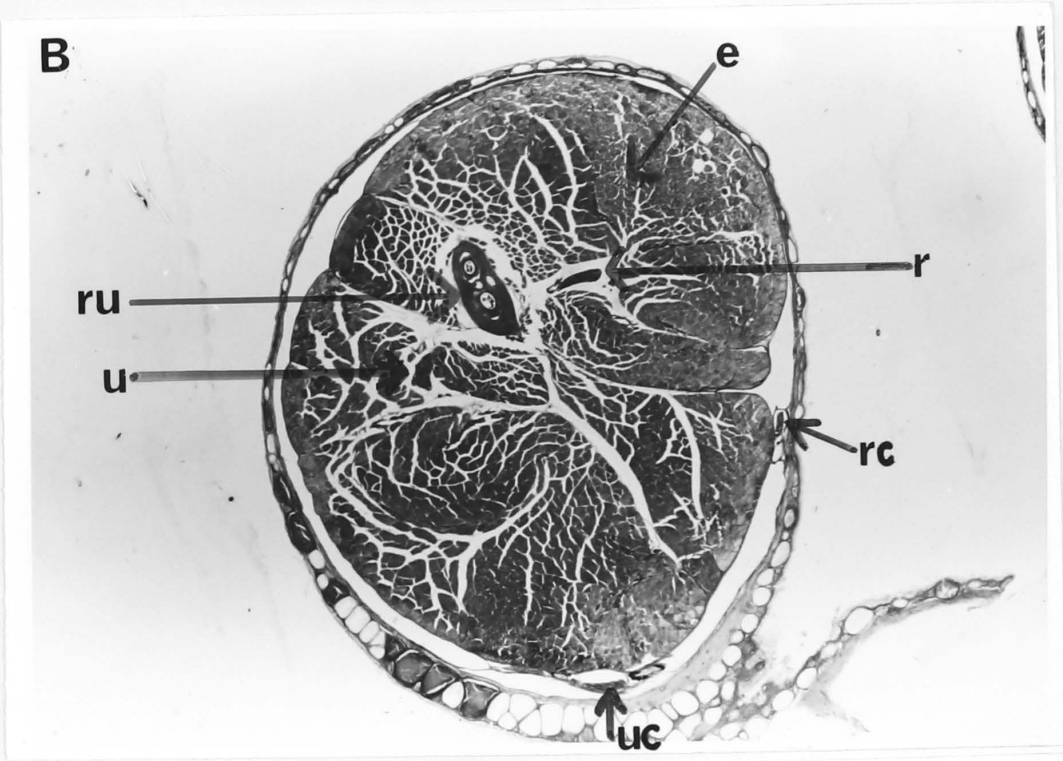
Abbreviations:

b	M. biceps
c	O. carpales
e	M. extensor digitorum communis
h	O. humerus
r	N. radialis
rc	N. radialis-ramus cutaneus superior
rl	N. radialis lateralis
rm	N. radialis medialis
ru	O. radio-ulnar
sr	sternoradialis tendon
t	M. triceps
u	N. ulnaris
uc	N. ulnaris-ramus cutaneus superior

FIG 2.7



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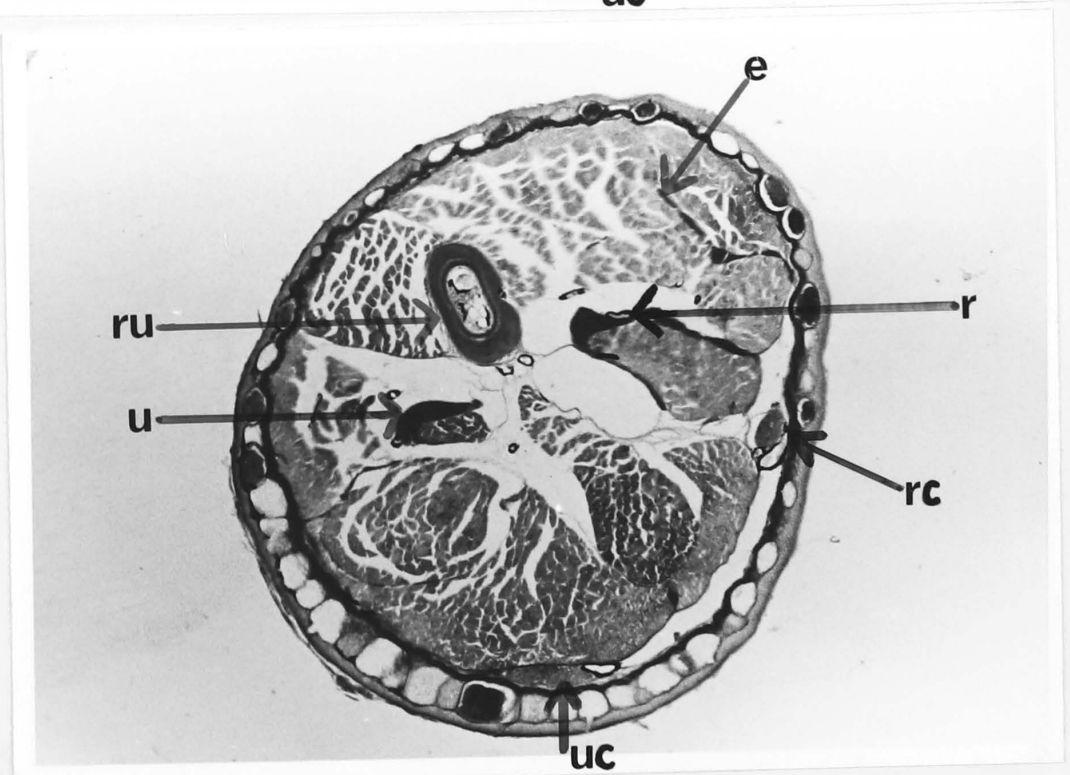
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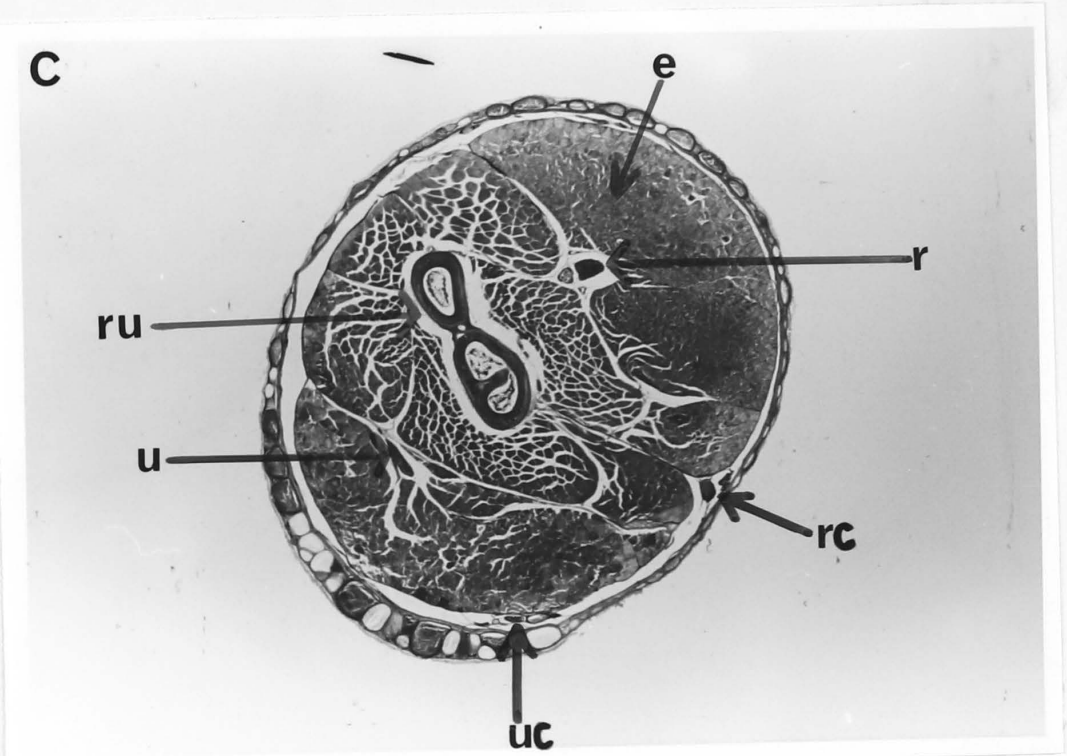
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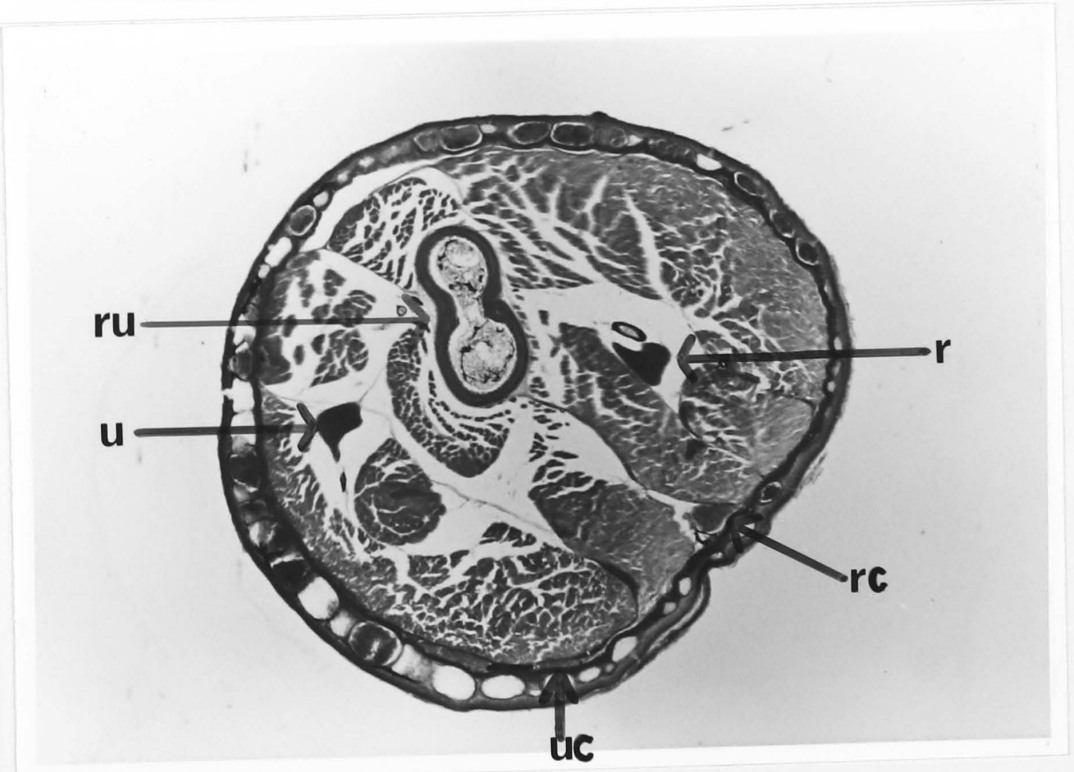
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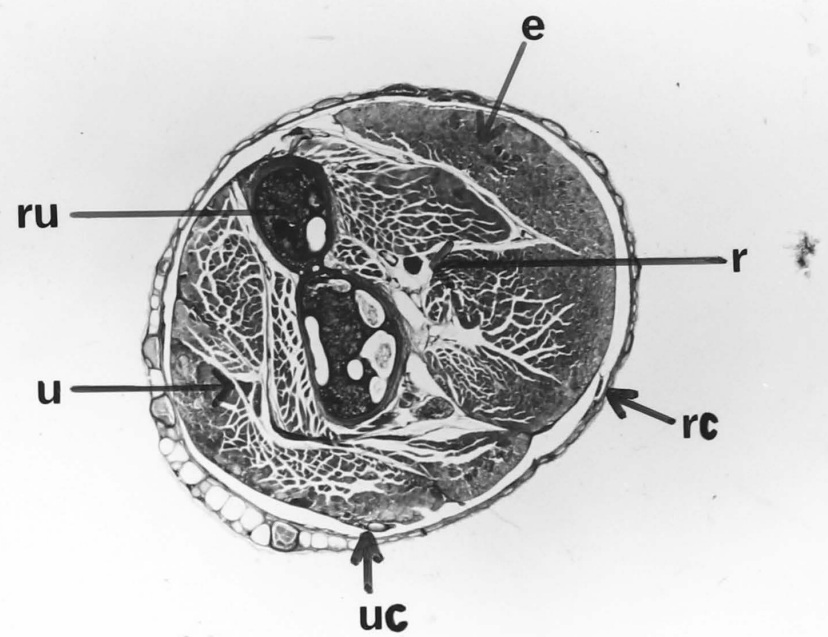

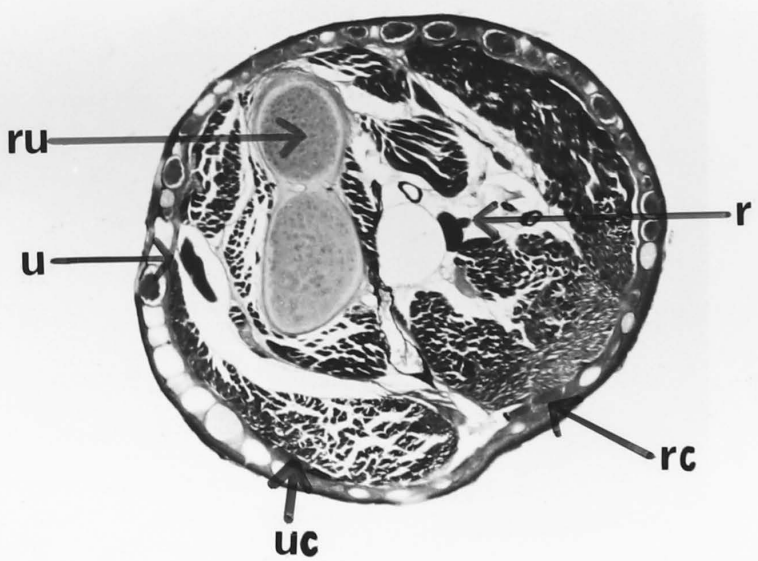

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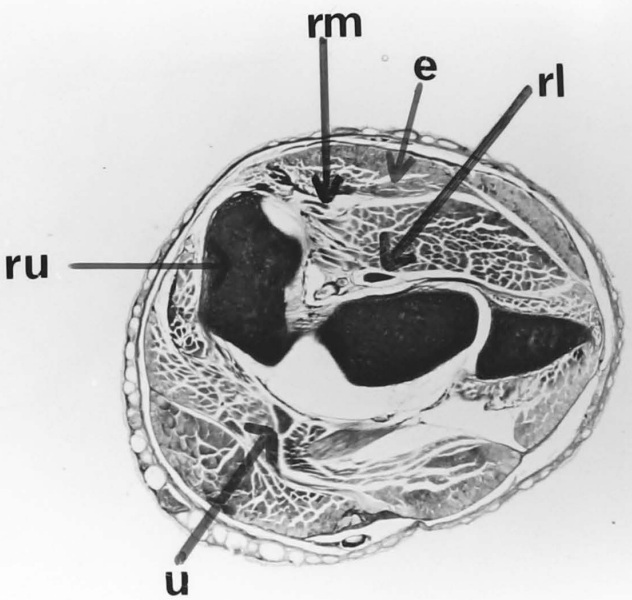

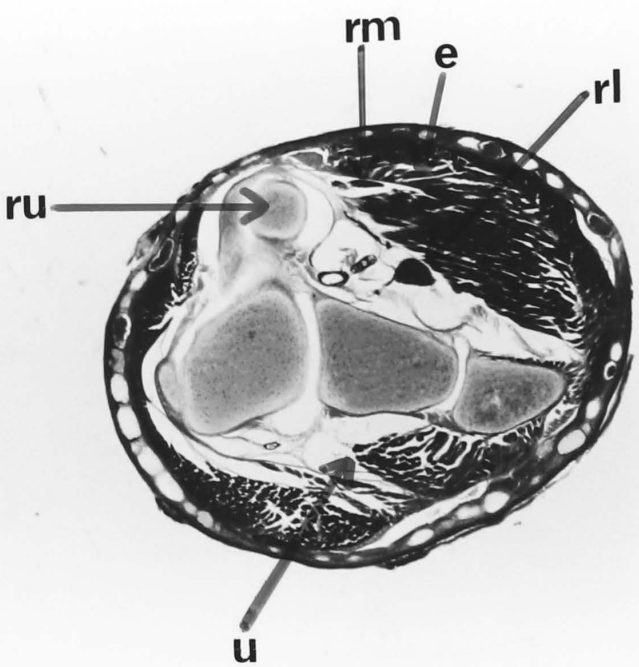

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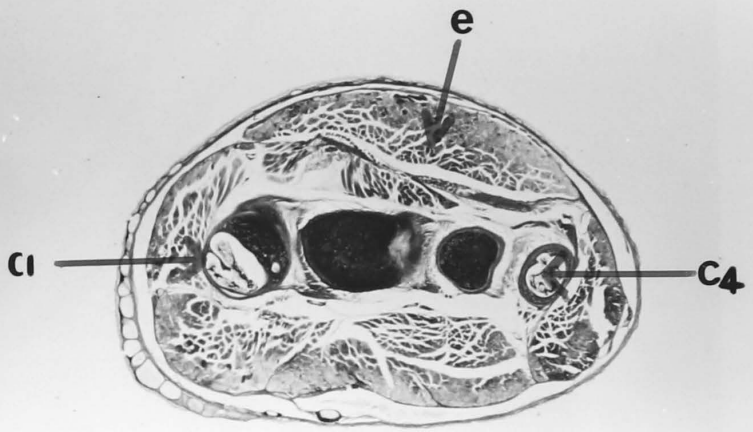

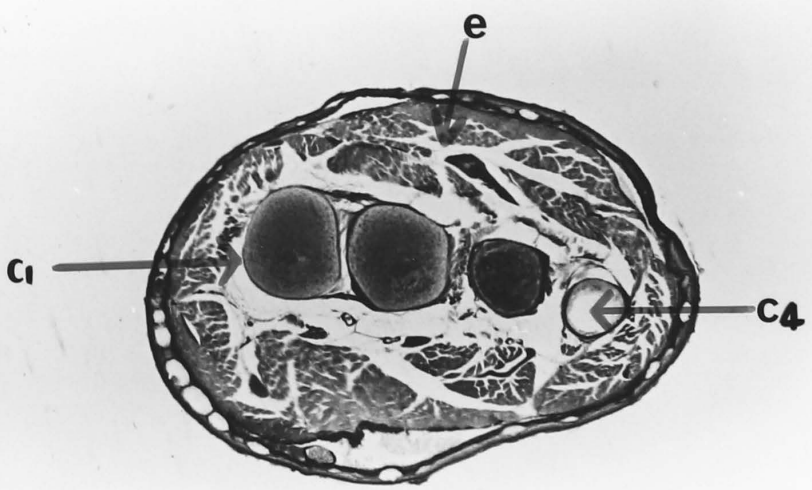

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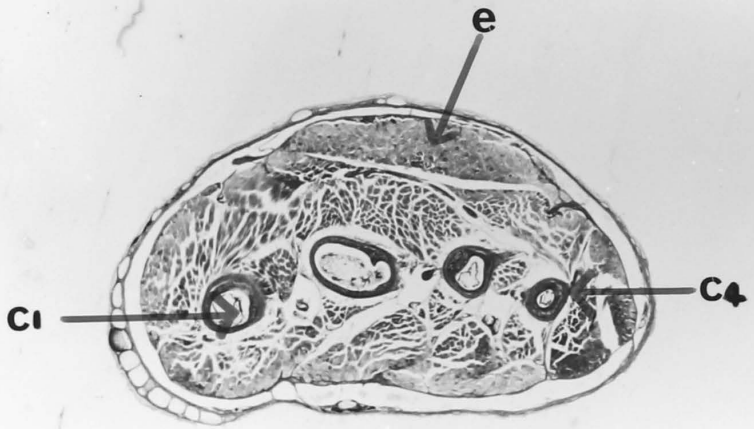
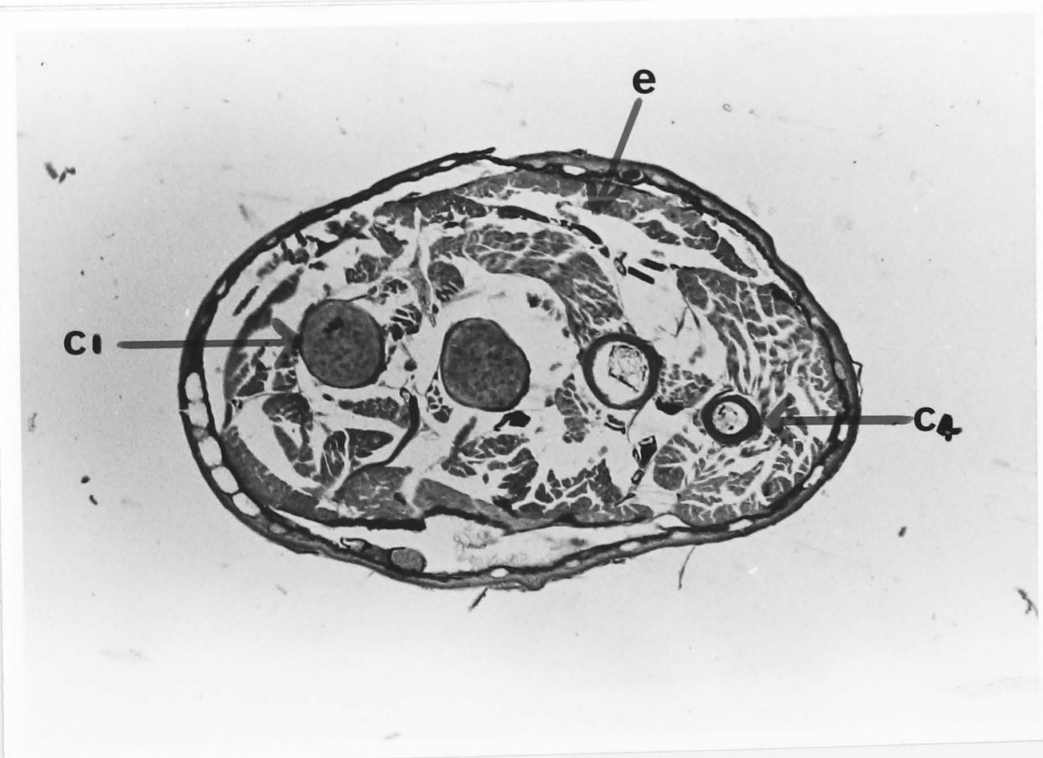




branches, before dividing at the level of the wrist (Fig. 2.7 (d)). These divisions passed branches to intrinsic muscles of the hand and fingers. In none of the limbs examined did the nerves follow abberant courses after entering the limb. The pattern was normal in both left and right-handed limbs supplied by the right-hand-side segmental nerves.

2.4 DISCUSSION

Supply of nerves to the Limb: Each transplanted forelimb received 3 nerves from the lumbar segments. The limb motor axons normally follow the same course as the motor axons to the myotomes, then depart towards the base of the limb (Taylor, 1943; Prestige and Wilson, 1980). This outgrowth does not require a limb to be present, since limb amputation prior to axon outgrowth results in a neuroma forming near the amputation site (Oppenheim, Chu-Wang and Maderdrut, 1978).

There is substantial evidence however, that the limb attracts the first axons during the final stages of their outgrowth, since heterotopic limbs acquire nerves from adjacent spinal segments, even at non-limb levels (Detwiler, 1936; Hamburger, 1939 a; Hollyday and Mendell, 1975; Morris, 1978).

The present study, in agreement with these earlier works, suggests that the attraction is relatively unspecific. In fact Detwiler (1936) found that spinal nerves deviated caudally towards a grafted optic vesicle or nasal placode, and he suggested that any rapidly growing tissue would attract nerves.

More recently it has been proposed that condensing mesenchyme in the dorsal and ventral muscle masses may exert a chemotactic attraction to motor axons (Bennett, Davey and Uebel, 1980), which in conjunction with the temporal sequence of delivery of axons to the limb, would determine the pattern of the limb nerve plexus. This is reflected in the nerve pattern found in the adult frog, which basically consists of 2 main mixed nerve trunks supplying the flexor and extensor musculature, and 2 main cutaneous nerves, supplying the dorsal and ventral skin, respectively, of the arm. The patterning of nerves upon entry and growth into the limb must involve other factors, however. Taylor (1944) found a normal branching pattern of sensory nerves in the limb, after extirpation of the ventral half of the spinal cord, containing the motoneurons. Limbs may also develop normal cutaneous and mixed nerve trunks following x-irradiation of the somitic mesoderm, which gives rise to the limb muscles (Lewis, Chevallier, Kieny and Wolpert, 1981). It is not yet clear whether sensory axons simply grow along the motor axons into the limb. This possibility is suggested by the emergence of cutaneous and proprioceptive reflexes *after* the onset of limb mobility (Windle and Orr, 1934; Hughes and Prestige, 1967), however this delay could also be due to the known retrograde sequence of synapse formation onto the motoneurons (Oppenheim, Chu-Wang and Foelix, 1975).

Pattern of Branching of the Limb Nerves: In the present study it was found that lumbar nerves, upon entering a transplanted forelimb bud, adopted a pattern of nerve branching normal to the limb. The abnormal nerve patterns observed by Piatt (1952, 1956) may have resulted from malformations of the limbs, as a high incidence of malformed limbs arose in the current series.

These observations raise questions concerning the initial formation of the nerve branches, which must involve interactions between the axons and the local environment (Stirling and Summerbell, 1977, 1979). The first axons enter the frog (Taylor, 1943; Prestige and Wilson, 1980), chicken (Bennett, Davey and Uebel, 1980) or rabbit (Cameron and McCredie, 1982) limb bud when its mesenchyme is undifferentiated. The delivery of axons therefore takes place during morphogenesis of the limb, and axons entering the limb bud at different times will face different environments.

The structures of the limb are determined in a proximo-distal sequence during limb morphogenesis. For example, in *Xenopus*, the first motor axons enter the hemispherical limb bud at stage 50 (Lamb, 1974) when only part of the pelvic girdle has been determined. At stage 51, when about half the total number of motoneurons have been generated, only the bones proximal to the knee are determined (Tschumi, 1957). Motoneuron death begins at stage 54 (Hughes, 1961; Prestige, 1967 a, 1970), by which time histogenesis of the musculature has begun (Muntz, 1975). At stages 51-52, the major nerve trunks are recognizable in the hindlimb of *Rana*, but during the early stages

the axons ramify widely within the mesenchyme (Taylor, 1943). The axons are bundled into nerve trunks by the processes of Schwann cells, which migrate after the growing front (Prestige and Wilson, 1980). These observations together suggest that the early growing axons are drawn into bundles by the same morphogenetic processes which determine the limb structures, rather than selectively growing along differentiated pathways, or following axial cues. Later arrivals will then grow into the pre-existing trunks.

Implications for the Development of Limb Innervation: Lance-Jones and Landmesser (1981 a, b) have suggested that motor axons may sort out on the basis of positional cues in the region of the limb plexus. For example, the flexor and extensor muscles of the hand are supplied by trunks that separate near the shoulder, and the axons to each muscle group would be required to discriminate between branches at that level. Indeed, Prestige and Wilson (1980) found that individual axons branched within the region of the plexus, and the finding that the pattern of nerve branching is determined by the limb, in itself does not counter the possibility of selective outgrowth of axons along those paths.

Motor axons will follow abberant paths in duplicated (Lewis, 1978) or rotated (Stirling and Summerbell, 1979; Summerbell and Stirling, 1981) limbs, however. There exists the alternative possibility that there is a selective *loss* of axons, following a period of unselective growth and termination within the limb.

2.5 SUMMARY

1. A stage 50 forelimb bud transplanted in place of a stage 49 hindlimb underwent excellent morphological and histological self-differentiation, in about 10% of operations. All limbs that completed development contained a shoulder joint.

2. Developmental abnormalities included hindlimb regeneration, duplication of the distal structures of the forelimb, and both regeneration and duplication. Duplication of hindlimbs transplanted in the forelimb position was also noted.

3. All transplanted limbs received innervation from 3 lumbar nerves, one more than the normal forelimb, and the nerves were reduced in diameter. The 3 nerves generally but not always formed a single trunk prior to entering the limb.

4. The entry point and branching pattern of the main mixed and cutaneous nerves was typical of a normal left or right forelimb.

CHAPTER THREE

THE PROJECTION FROM THE LUMBAR SPINAL CORD TO A TRANSPLANTED FORELIMB IN *XENOPUS*

II. NUMBERS AND SIZES OF MOTOR AND DORSAL ROOT GANGLION NEURONS

3.1 INTRODUCTION

Shorey (1909) noted that amputation of frog or chicken limb buds resulted in hypoplasia of the associated motor centre of the spinal cord. The hypoplasia was attributed to the death of young motoneurons (Hamburger, 1958; Prestige, 1967 a, 1970; Oppenheim, Chu-Wang and Maderdrut, 1978). It was later found that motoneurons also die during normal development. At the onset of limb mobility a period of neuron death begins, which ultimately eliminates over half the number of motoneurons initially present (Hughes, 1961; Hamburger, 1975; Fortune and Blackler, 1976; Oppenheim, Chu-Wang and Maderdrut, 1978; Oppenheim and Majors-Willard, 1978). Since almost all the motoneurons send an axon to the developing limb bud (Prestige and Wilson, 1974; Chu-Wang and Oppenheim, 1978 b) it is widely accepted that the death of motoneurons during normal development reflects the failure of some neurons to form peripheral connections, or an inability to sustain those already formed.

It was first suggested by Hughes (1968) that motoneuron death might serve to eliminate motoneurons which received the least appropriate central connections for the muscle in which their axons terminated. This suggestion was based on 3 lines of evidence, namely, the central determination of the motor centres of the spinal cord (Székely, 1963), a random element in the outgrowth of motor axons to the limb (Hughes and Prestige, 1967, Hughes, 1968), and a loss or turnover of motor connections during development (Hughes, 1961, 1964 a). Subsequent work has provided more substantial evidence

for early diffuse projections of motoneurons in the *Xenopus* hindlimb (Lamb, 1976), axolotl hindlimb (McGrath and Bennett, 1979) and chick forelimb (Pettigrew, Lindeman and Bennett, 1979).

In addition to the evidence compiled by Hughes (1968) a fourth factor has emerged in the phenomenon of motoneuron death, that of competition. The possibility of competition between motoneurons was first suggested by reinnervation experiments in urodele amphibia. It was found that after cutting the limb nerves, proper co-ordination of the limb muscles was eventually restored, not by respecification of central connections onto the motoneurons (Weiss, 1937 a, c, d; Sperry, 1941) but by motor nerves regaining control of their original territories (Cass, Sutton and Mark, 1973; Cass and Mark, 1975; Bennett and Raftos, 1977). This depended at least partly on competition between motoneurons at the neuro-muscular junctions (Yip and Dennis, 1976; Dennis and Yip, 1978; Bennett, McGrath and Davey, 1979; Wigston, 1979). Thus, it remains to be established if motoneurons die in normal development because they fail to contact appropriate limb regions, or because they lose in competition with neurons more appropriate for a particular limb region, or both (Lamb, 1981 b). In the experiments here, the hindlimb bud of *Xenopus* tadpoles was replaced with that of a forelimb, or several myotomes prior to axon outgrowth from the spinal cord. The surviving motor and dorsal root ganglion neurons were counted in the lumbar segments after metamorphosis, and the sizes of the motoneurons measured.

3.2 METHODS

Embryology: Forelimb transplantations were performed as described in the Methods for Chapter 2. In other animals a hindlimb bud was transplanted in place of the forelimb bud. Transplantation of myotomes took place under the same operating conditions. Several myotomes were obtained from the mid-tail level of a stage 49 donor, and this tail section was bissected from the dorsal to the ventral fin. Each hemisection, containing several hundred muscle fibres, was transplanted in place of the hindlimb bud of a stage 49 host. Great care was taken in removing the skin covering the abdominal muscle fibres. As a control, a group of tadpoles had one hindlimb bud amputated at stage 49, and re-amputated after any regeneration.

Histology: For cell counts and nuclear area measurements, animals ranging from 52 days (Stage 63, Nieuwkoop and Faber, 1967) to 1½ years of age were used. Young animals were preferred since the lack of pelvic bones and musculature on the operated side led to a progressive deformation of the vertebral column after metamorphosis, with the added difficulty of obtaining a uniform plane of section.

Animals were anaesthetized in MS222 solution. The largest were perfused through the heart with 0.65% NaCl at 4°C then immersed and dissected in 37.5% saturated picric acid, 12.5% formalin, 2.5% glacial acetic acid. It was not possible to remove the vertebrae without damage to the dorsal root ganglia, so they were left intact. The spinal columns were left in fixative for at least

48 hours, while the picric and acetic acids de-calcified the vertebrae sufficiently for sectioning. Cords were dehydrated in ethanol, with three changes of 2 hr at 90%, then three changes of 1 hr at 100%, then cleared in three changes of methyl benzoate followed by infiltration in two changes of Tissue Prep. embedding medium (melting point 54°C - Fisher Sci. Co., New Jersey) at 60°C and two atmospheres pressure. Serial 12µm thick transverse sections were cut and mounted on glass slides coated with gelatin. They were re-hydrated, stained for Nissl substance with cresyl violet (Schmid GMBH and Co., Stuttgart), dehydrated in 95% and 100% ethanol, cleared in xylene, and mounted under glass coverslips with Depex, or Eukitt (Vitromed, Switzerland) media.

Cell counts: The comparatively small number of motoneurons supplying the *Xenopus* hindlimb enabled them to be counted in every section. Each cell in the ventrolateral cord with a single large nucleolus surrounded by a regular unbroken nuclear outline much larger than other cells was counted as a motoneuron (Hughes, 1961). Dorsal root ganglion neurons were present in greater numbers than the motoneurons, and were therefore counted only in every third section. Each cell with a single large nucleolus, surrounded by a regular unbroken nuclear outline and stained cytoplasm, was counted as a neuron. This distinguished them from interstitial and satellite cells, and possibly neuroblasts in the youngest animals (Prestige, 1965).

Error estimates in cell counts

Counting errors may arise due to a cell being missed or counted twice. To estimate this, motoneurons on both sides in 30 sections were counted on 5 successive days, the totals being 170, 168, 170, 172 and 173. The standard error of counts as a percentage of the mean count was therefore estimated to be less than 2%. An error may arise when a single nucleolus is sectioned and counted in 2 adjacent sections. Assuming spherical nucleoli, the correction of Abercrombie (1946) was applied to the total cell count as follows:

$$\text{Corrected Total } N = n\left(\frac{T}{T+D}\right)$$

where

n = actual total

T = section thickness

D = mean nucleolus diameter.

Nuclear area measurement: In every 4th section an outline of each motoneuron nucleus enclosing a nucleolus was drawn with the aid of a camera lucida attached to an Orthoplan II microscope (Leitz Wetzlar, Germany). The final magnification of 1250 with a 100X oil immersion objective and 10X eyepiece gave a nucleus image of about 1 cm diameter at the plane of the microscope base. Nuclear outlines were digitized using a Tektronic 4663 Interactive Digital Plotter run on a program by a Tektronix 4054 graphic computer, which calculated the area enclosed by each nuclear outline.

Errors in nuclear area measurements: In the statistical comparison of nuclear diameters it was assumed that systematic errors caused by variations such as fixation shrinkage or obliquity of the plane of sectioning, were the same on both sides of the spinal cord. As an estimate of the error intrinsic to the measurement procedure, a circle of 1 cm final diameter was traced from the microscope 10 times and the areas measured as described above. The standard error of area measurement as a percentage of the mean was estimated to be 3% in this case.

Notes on the Normal Innervation of the Hindlimb: The system of Gaupp (1896) is used here in numbering the spinal nerves. In this system, spinal nerve 1 is lost prior to metamorphosis, leaving nerves 2 (hypoglossal), 3 etc. The hindlimb plexus is most commonly supplied by nerves 8, 9, 10 and 11. Nerve 7 may occasionally contribute (Prestige and Wilson, 1974). The dorsal root ganglia (DRG) supplying the hindlimb consistently differ in size with $11 << 10 \geq 9 > 8$. The dorsal roots enter the dorsal funiculi at discrete locations whereas the axons that form the ventral roots emerge along almost the entire length of each segment.

The lateral motor column increases in length with the growth of the animal, from about 1200μm prior to metamorphosis to over 3000μm in the adult (Hughes, 1961). During this time the DRG descend with respect to their roots, so that DRG 8 may lie within the region of entry of 10 (Nieuwkoop and Faber, 1967). The lateral motor column arises anterior to root 8 (Fig. 3.2), and extends posterior to root 10. The medial column is present in all segments except 8 and 9.

The motoneurons increase in number per section passing caudally through the LMC, reaching a peak density in segment 10, then rapidly declining (Silver, 1942; Cruce, 1974 a). Motoneurons are somatotopically organized within the LMC. In general thigh motoneurons are located rostrally, shank and foot motoneurons are located caudally, and flexor motoneurons are more medial than extensor motoneurons at the same rostrocaudal level (Cruce, 1974 a; Lamb, 1976).

3.3 RESULTS

Development of transplanted Forelimbs: A number of transplanted limb buds completed self-differentiation in the hindlimb position, and suppressed hindlimb regeneration (Fig. 3.1 (a), (b)). All of these limbs were morphologically and anatomically normal, included a shoulder joint, and were mobile and sensitive to cutaneous stimuli. The lumbar nerve supply was from only the ipsilateral side, as judged by dissection (Chapter 2), histological sections and horseradish peroxidase labelling of motoneurons (Chapter 4).

Lumbar motoneuron distributions and totals following transplantation of a Forelimb bud in place of the Hindlimb:

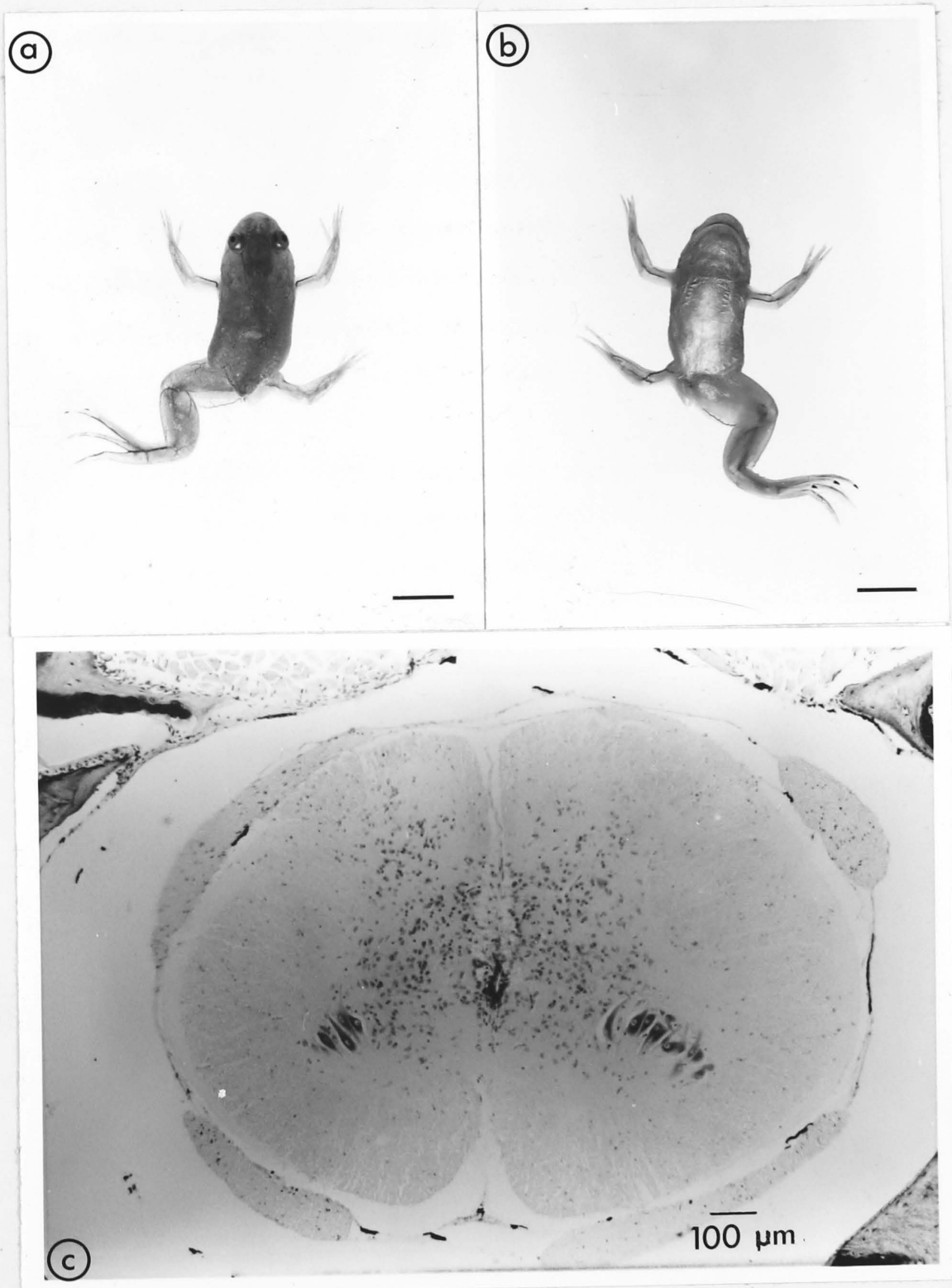
The lumbar motoneurons of *Xenopus* were conspicuous in histological sections, and there was a relatively uniform reduction in neuron numbers on the operated side (Fig. 3.1 (c)) with totals ranging from 30 to 56% (mean 46%) of the numbers on the unoperated side (Table 3.1).

FIGURE 3.1 FROG WITH A TRANSPLANTED FORELIMB IN THE HINDLIMB POSITION

(a) Animal J, Bar = 5mm.

(b) Animal J, Bar = 5mm.

(c) Cross-section of the spinal cord of J at the level of entrance of dorsal root 9 - the operated side is on the left. The ventral root bundle is smaller on the operated side


a
b
c
100 µm

This was reflected in the cross-sectional area of the ventral roots (Fig. 3.1 (c)).

The LMC on the operated side was of normal length (Figs. 3.2, 3.3), and the motoneurons were distributed through most sections. The distribution reflected that of the normal side, except that in some animals (F and H) the caudal peak in segment 10 was not evident. Animal H had no movements of the hand during swimming.

In most animals the DRG on the operated side were normal in terms of relative sizes (10>9>8) except in animal H, where ganglion 10 was unusually small. In terms of neuron numbers the ganglia were reduced in size, but not by as great an amount as with the motoneurons (Table 3.2).

TABLE 3.1 LUMBAR MOTONEURON TOTALS FOLLOWING TRANSPLANTATION OF A FORELIMB BUD IN PLACE OF THE HINDLIMB*

ANIMAL	LMC LENGTH (μm)	MOTONEURON - TOTALS (corrected) OPERATED SIDE	UNOPERATED SIDE	COUNT CORRECTION FACTOR	OPERATED/ UNOPERATED %
A	2208	354	799	0.92	47
B	2100	369	897	0.91	41
C	1380	401	885	0.94	45
D	1512	392	868	0.93	45
E	1884	389	995	0.88	39
F	2112	406	972	0.89	42
H	3456	500	1293	0.87	39
I	2700	526	940	0.89	56
J	3504	542	986	0.88	55
K	3320	447	913	0.92	49
		4,326	9,498		46

*All animals past developmental stage 60.

FIGURE 3.2 SCHEMATIC DIAGRAM OF THE LUMBAR SEGMENTS, ANIMAL J

The lateral motor column extends through segments 8, 9 and 10. The medial motor column ends in segment 7, and recommences in the caudal half of segment 10.

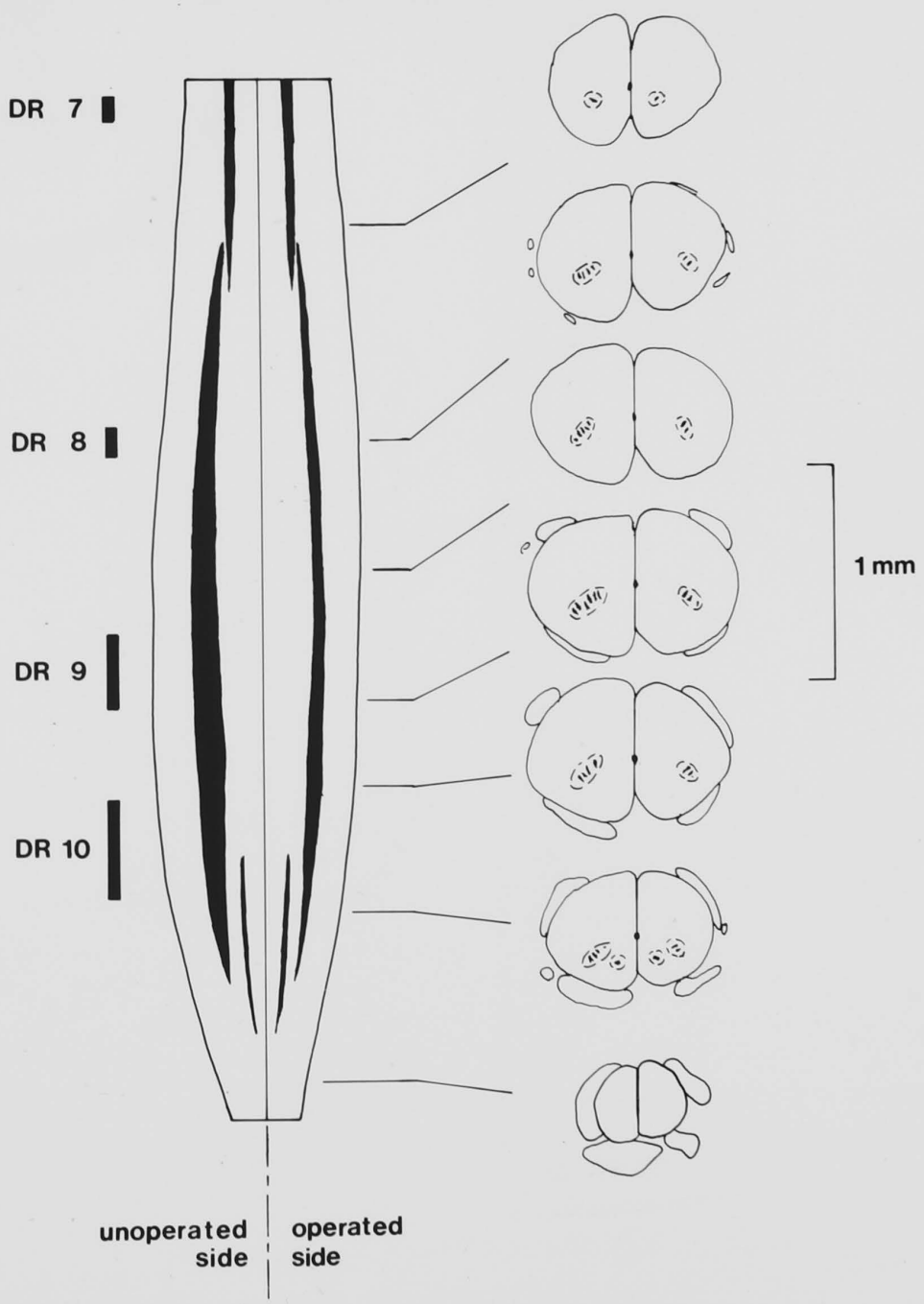
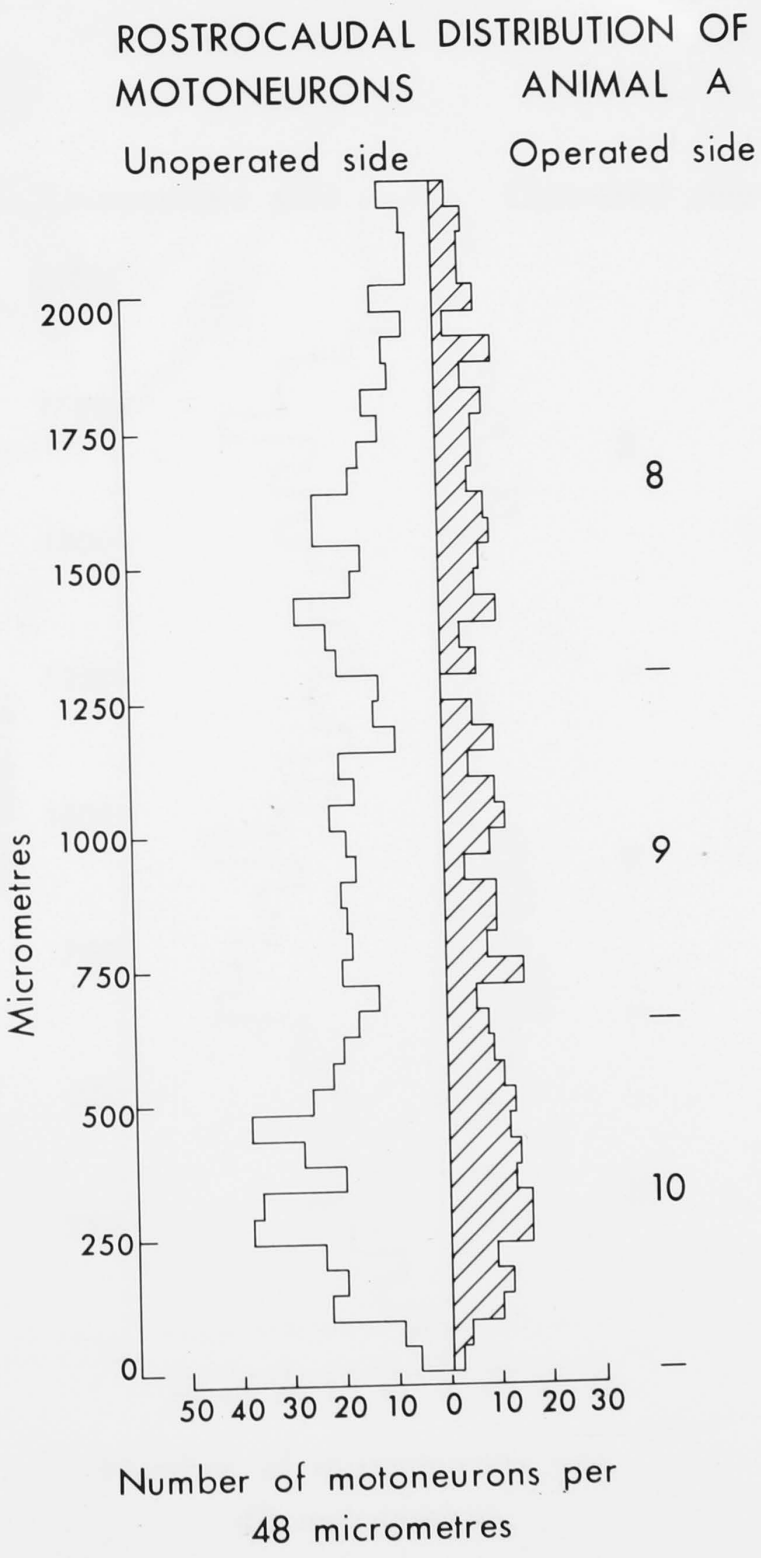
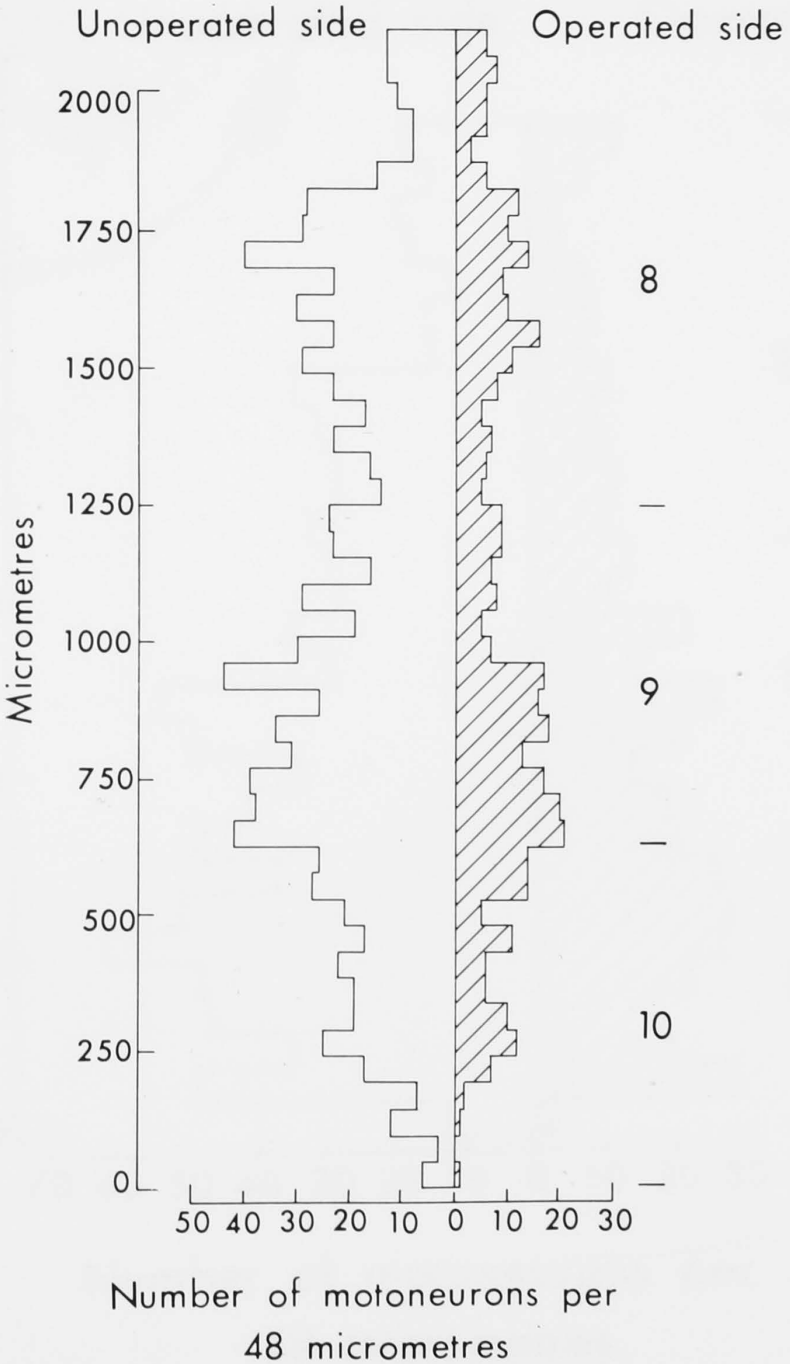

LUMBAR SEGMENTS
ANIMAL J
DR 7
DR 8
DR 9
DR 10
1 mm
unoperated side
operated side

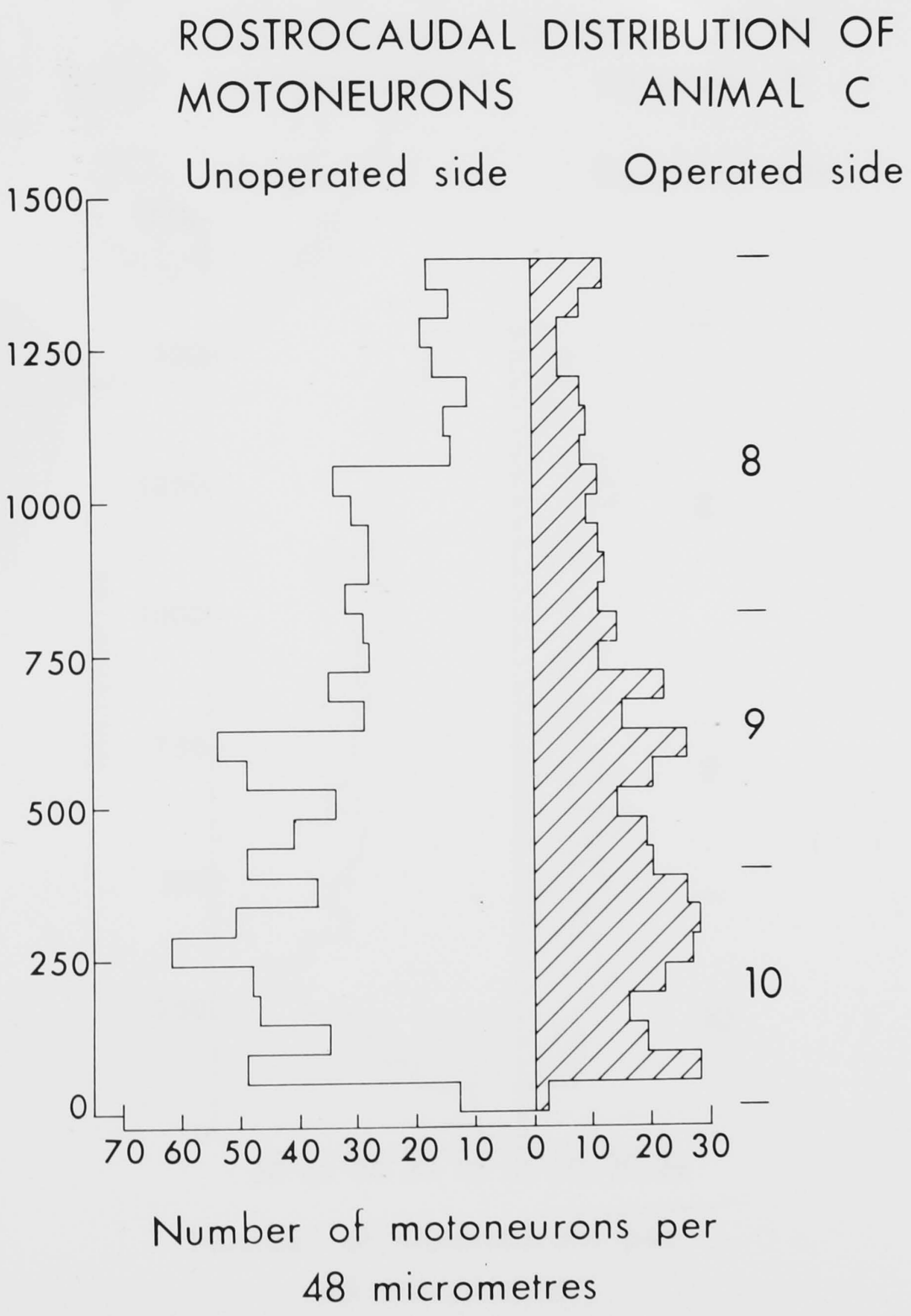
FIGURE 3.3 ROSTROCAUDAL DISTRIBUTION OF LUMBAR MOTONEURONS, ANIMALS A - K

Spinal cord segments were demarked by the dorsal root entry zones

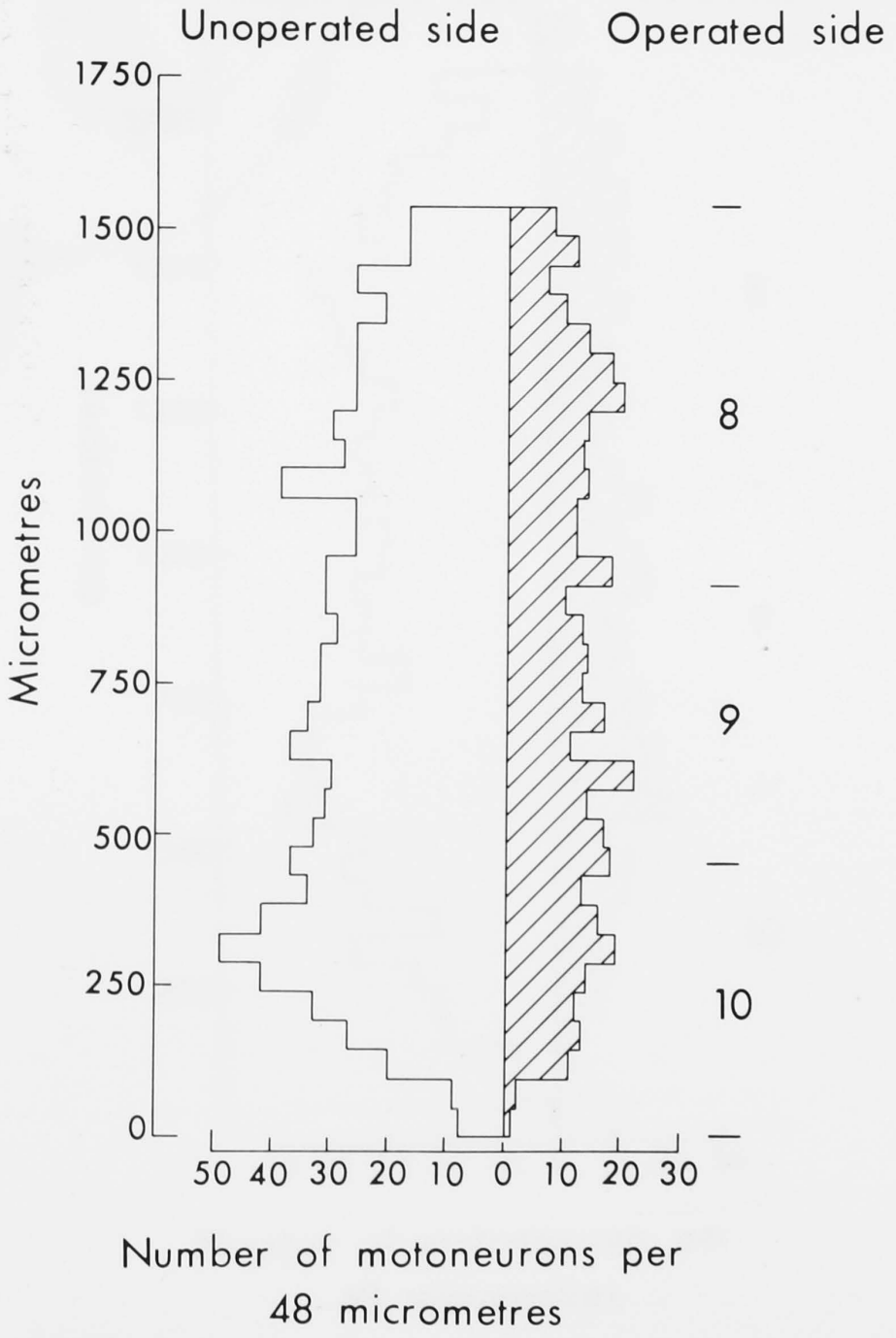

ROSTROCAUDAL DISTRIBUTION OF MOTONEURONS ANIMAL A
Unoperated side
Operated side
2000
1750
1500
1250
1000
750
500
250
0
Micrometres
8
9
10
50 40 30 20 10 0 10 20 30
Number of motoneurons per 48 micrometres

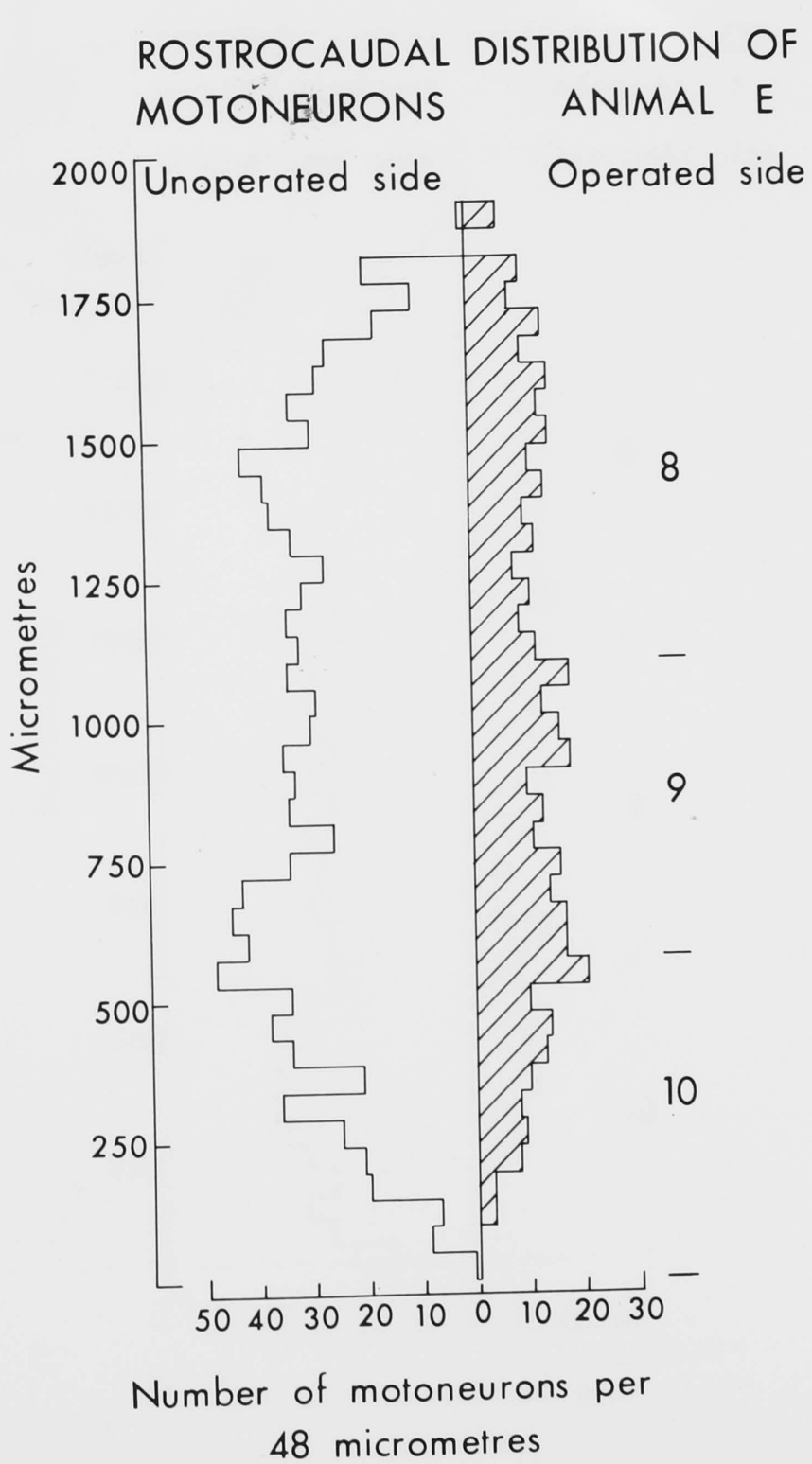
ROSTROCAUDAL DISTRIBUTION OF MOTONEURONS ANIMAL B



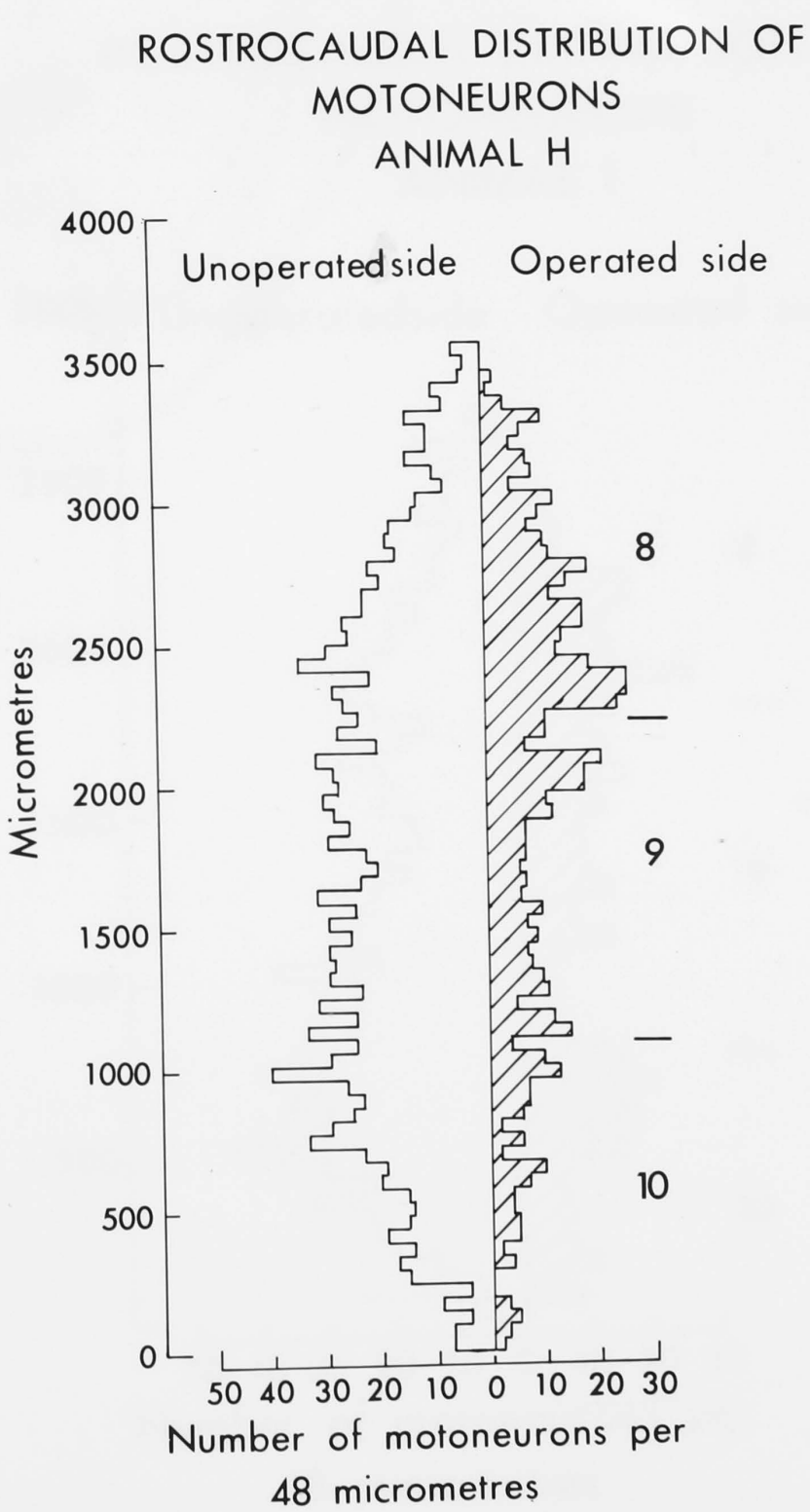

ROSTROCAUDAL DISTRIBUTION OF MOTONEURONS
ANIMAL C
Unoperated side
Operated side
1500
1250
1000
750
500
250
0
8
9
10
70 60 50 40 30 20 10 0 10 20 30
Number of motoneurons per 48 micrometres

ROSTROCAUDAL DISTRIBUTION OF MOTONEURONS ANIMAL D

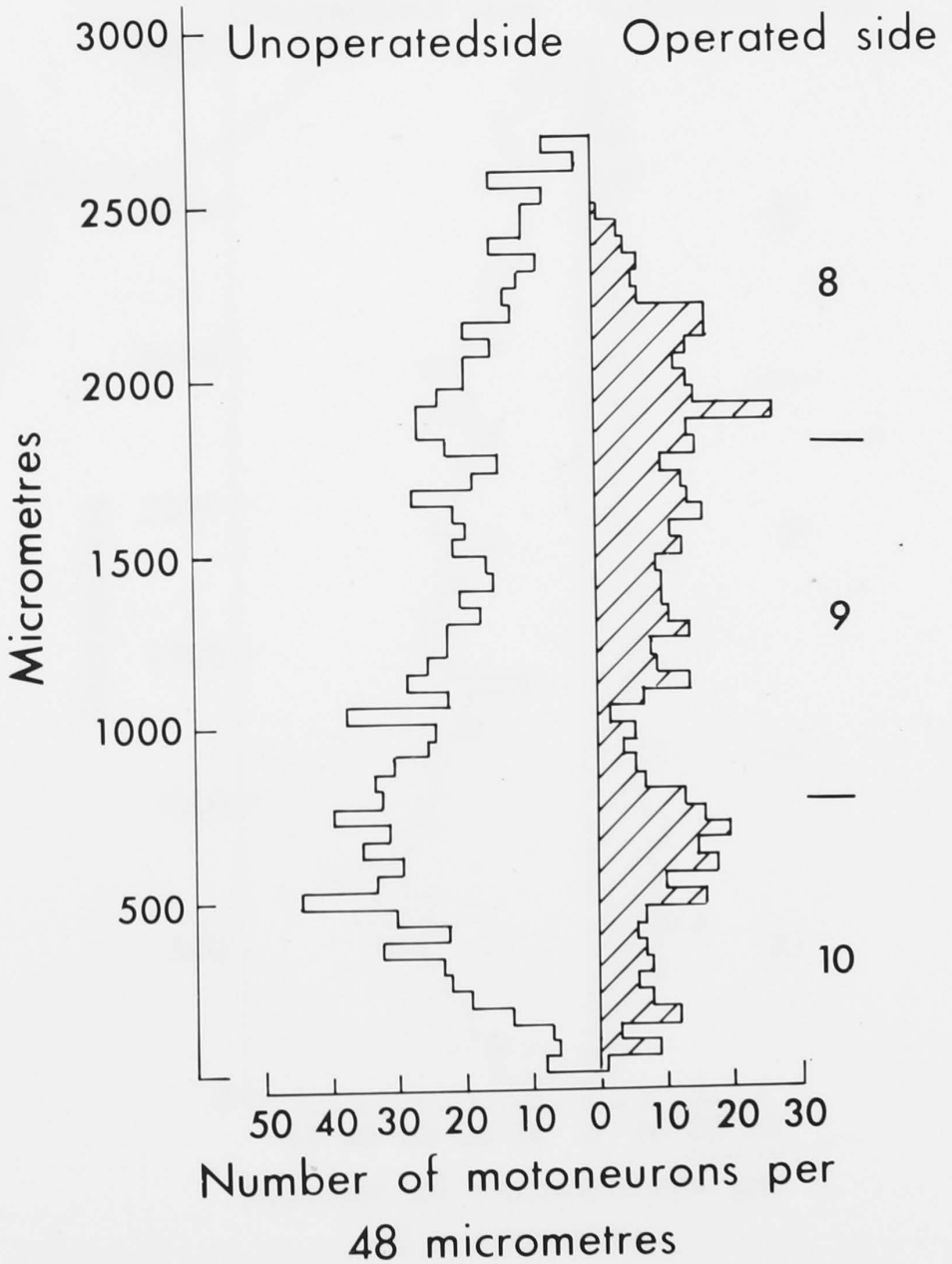



ROSTROCAUDAL DISTRIBUTION OF MOTONEURONS
ANIMAL E
Unoperated side
Operated side
2000
1750
1500
1250
1000
750
500
250
Micrometres
8
9
10
50 40 30 20 10 0 10 20 30
Number of motoneurons per 48 micrometres

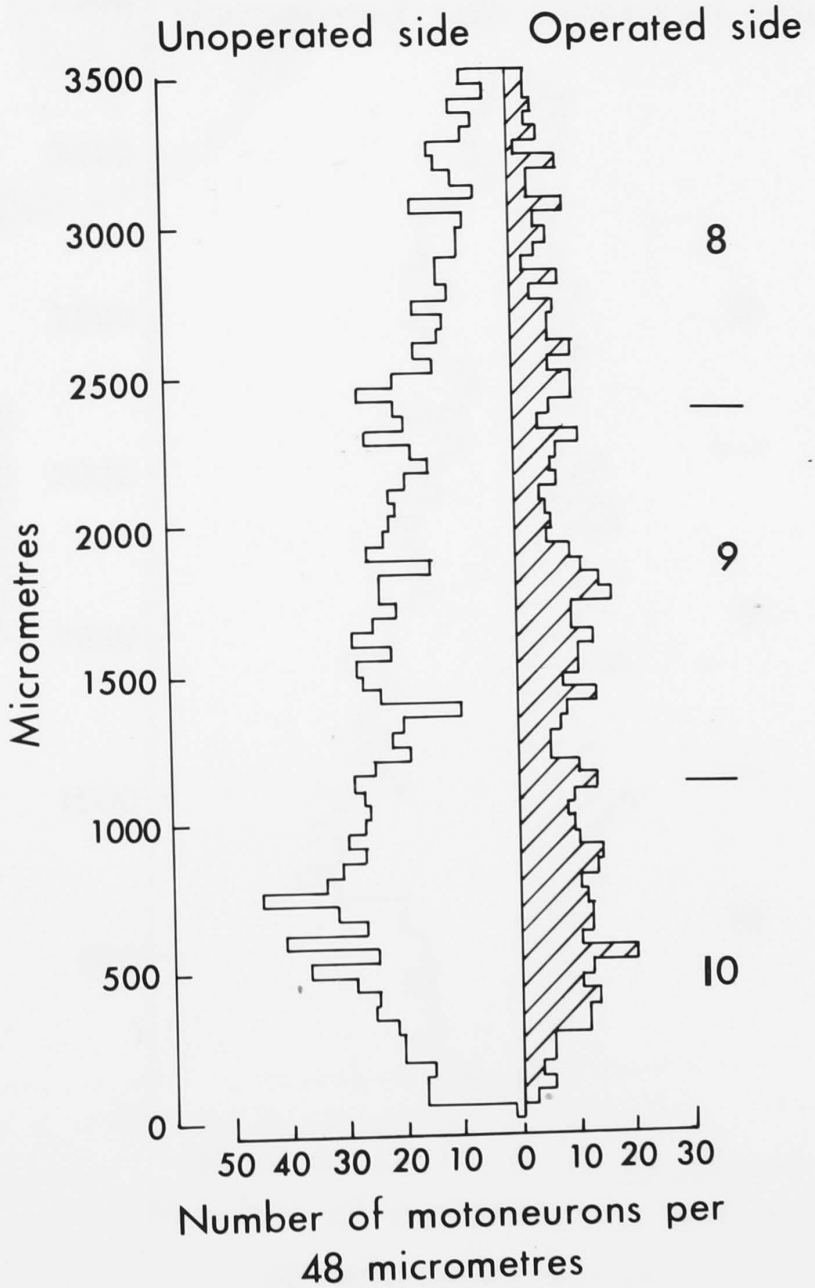

ROSTROCAUDAL DISTRIBUTION OF MOTONEURONS ANIMAL F
Unoperated side
Operated side
2000
1750
1500
1250
1000
750
500
250
Micrometres
8
9
10
50 40 30 20 10 0 10 20 30
Number of motoneurons per 48 micrometres


ROSTROCAUDAL DISTRIBUTION OF MOTONEURONS
ANIMAL H
Unoperated side
Operated side
Micrometres
4000
3500
3000
2500
2000
1500
1000
500
0
8
9
10
50 40 30 20 10 0 10 20 30
Number of motoneurons per 48 micrometres

ROSTROCAUDAL DISTRIBUTION OF MOTONEURONS
ANIMAL I



ROSTROCAUDAL DISTRIBUTION OF MOTONEURONS
ANIMAL J



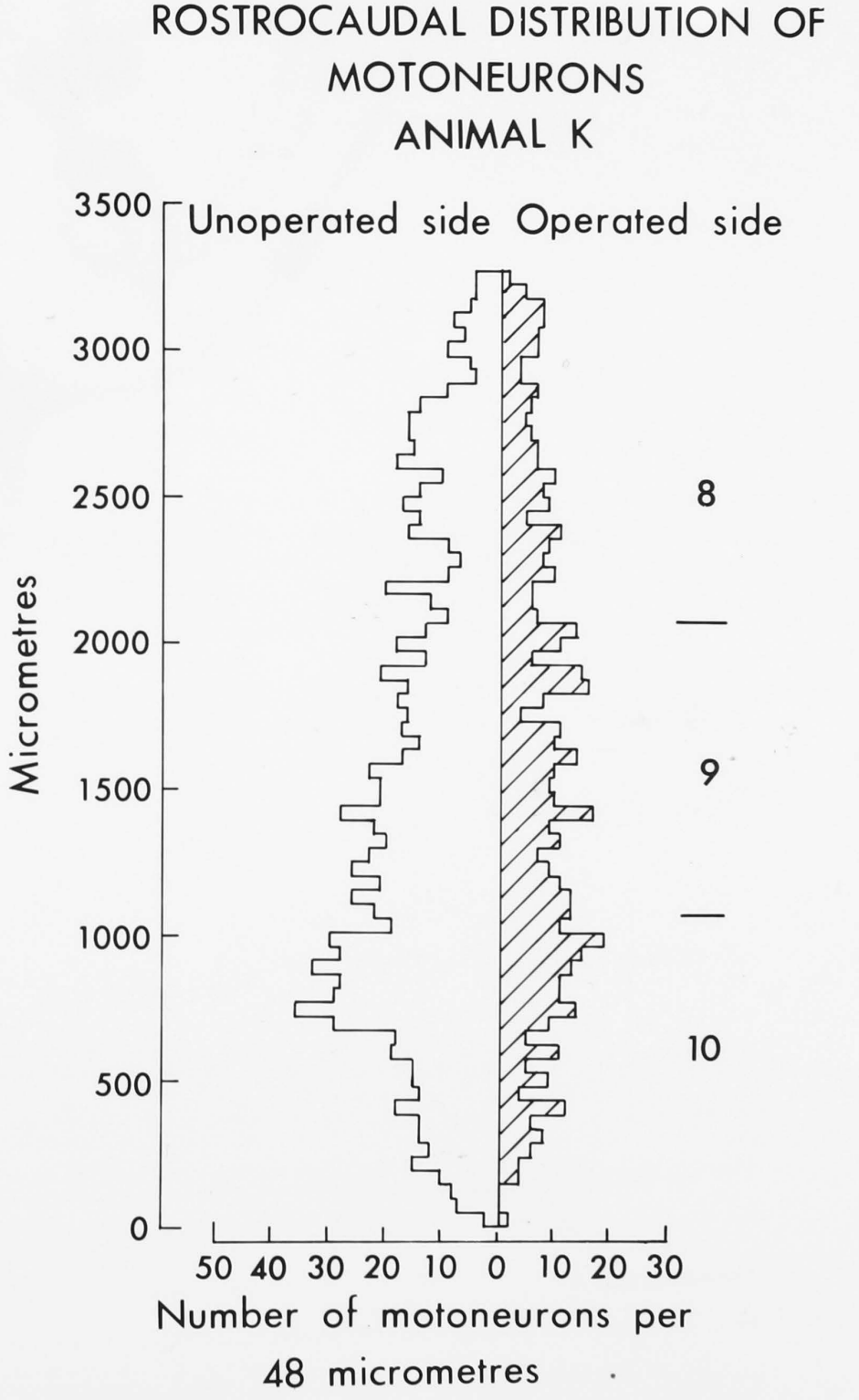

ROSTROCAUDAL DISTRIBUTION OF
MOTONEURONS
ANIMAL K
Unoperated side
Operated side
Micrometres
3500
3000
2500
2000
1500
1000
500
0
8
9
10
50 40 30 20 10 0 10 20 30
Number of motoneurons per
48 micrometres

TABLE 3.2 LUMBAR DRG 8+9+10 NEURON TOTALS FOLLOWING TRANSPLANTATION OF A FORELIMB BUD IN PLACE OF THE HINDLIMB

	DRG NEURON TOTALS (corrected)			
ANIMAL	OPERATED SIDE	UNOPERATED SIDE	COUNT CORRECTION FACTOR	OPERATED/ UNOPERATED %
A	4117	5208	0.91	79
B	4622	5143	0.99	90
C	4917	4728	0.90	104
H	3780	4573	0.90	93
I	3826	4473	0.90	86
J	4424	5431	0.87	81
	25,686	29,556		87

TABLE 3.3 LUMBAR MOTONEURON TOTALS FOLLOWING TRANSPLANTATION OF SEVERAL MYOTOMES IN PLACE OF THE HINDLIMB BUD

	MOTONEURON TOTALS (corrected)			
ANIMAL	OPERATED SIDE	UNOPERATED SIDE	COUNT CORRECTION FACTOR	OPERATED/ UNOPERATED %
M	39	982	0.89	4
N	60	992	0.90	6
O	166	1105	0.91	15
P	67	956	0.89	7
	332	4,035		8

Animals killed after metamorphosis

Development of transplanted myotomes: Successful transplants (Figs. 3.4 (a) (b)) acquired a blood supply within 48 hours, however many were subsequently lost during vigorous swimming movements, or by damage to the underlying abdominal musculature. Loss of a transplant at stages 49-54 was almost always followed by hindlimb regeneration. In some instances a hindlimb regenerated to one side of a transplant, eventually displacing it from the host. The regenerated hindlimb supported the normal total of motoneurons.

FIGURE 3.4 LIMB BUD REPLACEMENTS

(a) Stage 49 tadpole with several myotomes in place of the hindlimb

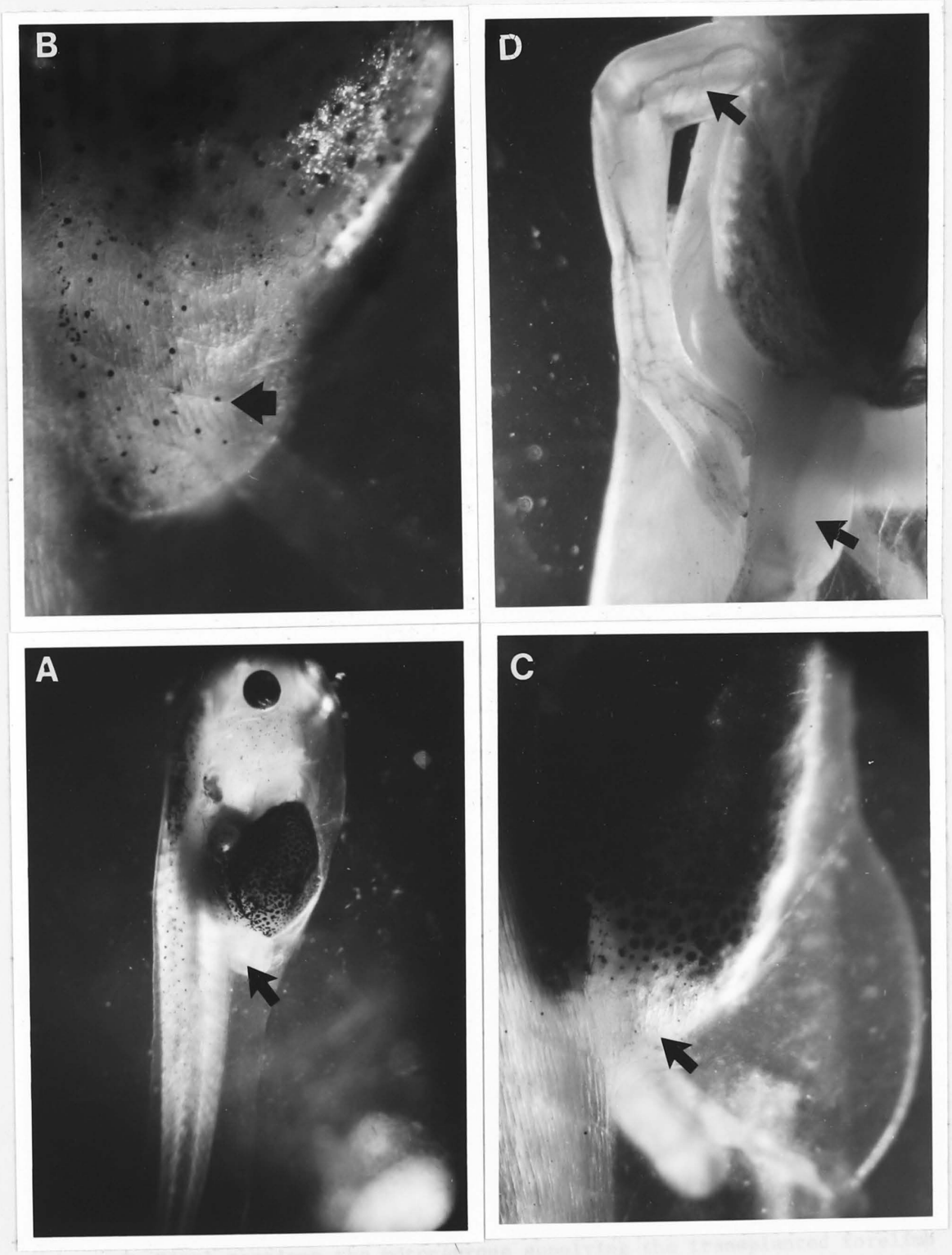
(b) Transplanted myotomes

(c) Regressing transplanted myotomes

(d) Hindlimb in place of the right forelimb. The limb is atrophic in comparison with the normal hindlimb

8 operations were successful. The stage 50 hindlimb bud was transplanted in place of the forelimb bud of a stage 49 recipient. The transplanted limbs developed normally, as judged by external appearance, although some formed duplicates, however towards metamorphosis the limb musculature appeared atrophic.

Regrettably the batch was lost due to accidental death prior to metamorphosis. The limb musculature and spinal cords were therefore not examined histologically.


B
D
A
C

The first motor axons enter the hindlimb bud at stage 50 (Lamb, 1974) and lumbar motoneuron death commences at stages 53-54 (Hughes, 1961). An increase in size of the transplant from stage 49 onwards was never observed, although the blood supply, once established, was always maintained. All transplants that remained in place over several stages of development showed fibrillations of their muscle fibres, and underwent complete atrophy before stage 58 (Fig. 3.4 (c)).

Lumbar motoneuron totals following transplantation of several myotomes in place of the Hindlimb

In 4 tadpoles out of a total of 78, the transplant remained in place during its complete regression, until no muscle fibres could be seen within. The lumbar notoneurons on both sides of the spinal cord were counted in these animals after metamorphosis, and also in a control group which had undergone unilateral amputation of the limb bud, which was repeated from stage 49 onwards, until regeneration ceased (Table 3.3). A small number of motoneurons were scattered through the lateral motor column (LMC) on the operated side, and they appeared normal in size and morphology. The total, however, was less than for the control group (N = 4, operated/unoperated 9%). It is likely that the few remaining motoneurons projected to the contralateral limb (Lamb, 1980, 1981, a).

Nuclear area measurement of lumbar motoneurons supplying the normal hindlimb, or transplanted forelimb:

In Nissl sections the motoneurons supplying the transplanted forelimb appeared normal (Fig. 3.5). The two main dendrites, extending dorsolaterally and ventromedially (Székely, 1976) were conspicuous

in some neurons. Each neuron contained an almost circular nucleus of about 10μm diameter, enclosing a single nucleolus.

At all stages of development there was a considerable range of nuclear areas of the motoneurons, on both the unoperated and operated sides of the spinal cord (Fig. 3.6). There was also a tendency for the smallest motoneurons to be the most ventrolateral within the LMC, although this was not investigated systematically. All the area distributions, with the exception of the hindlimb motoneurons of animal E were positively skewed and leptokurtic (Table 3.4). It was not possible to normalize all the distributions by a square root or logarithmic transformation. The distributions most likely reflected a heterogeneous neuron population, and comparisons of sizes in terms of mean values would not necessarily be valid (cf. Hollyday and Mendell, 1976). A Kolmogorov-Smirnov test was applied to the paired distributions, and a significant difference was found for animals D and I (Table 3.4). In the case of animal D the difference appeared to be due to the greater number of large neurons on the operated side, whereas for animal J the converse held.

Sizes of dorsal root ganglion neurons:

In contrast to the ganglia at non-limb levels, the lumbar DRG lack the large early-differentiating ventrolateral neurons (Hughes and Tschumi, 1958). DRG neurons on the operated side appeared normal (Fig. 3.5 (c), (d)), and the range of sizes, although not examined systematically, appeared to match that of the unoperated side.

FIGURE 3.5 LUMBAR NEURONS SUPPLYING A NORMAL HINDLIMB AND TRANSPLANTED FORELIMB

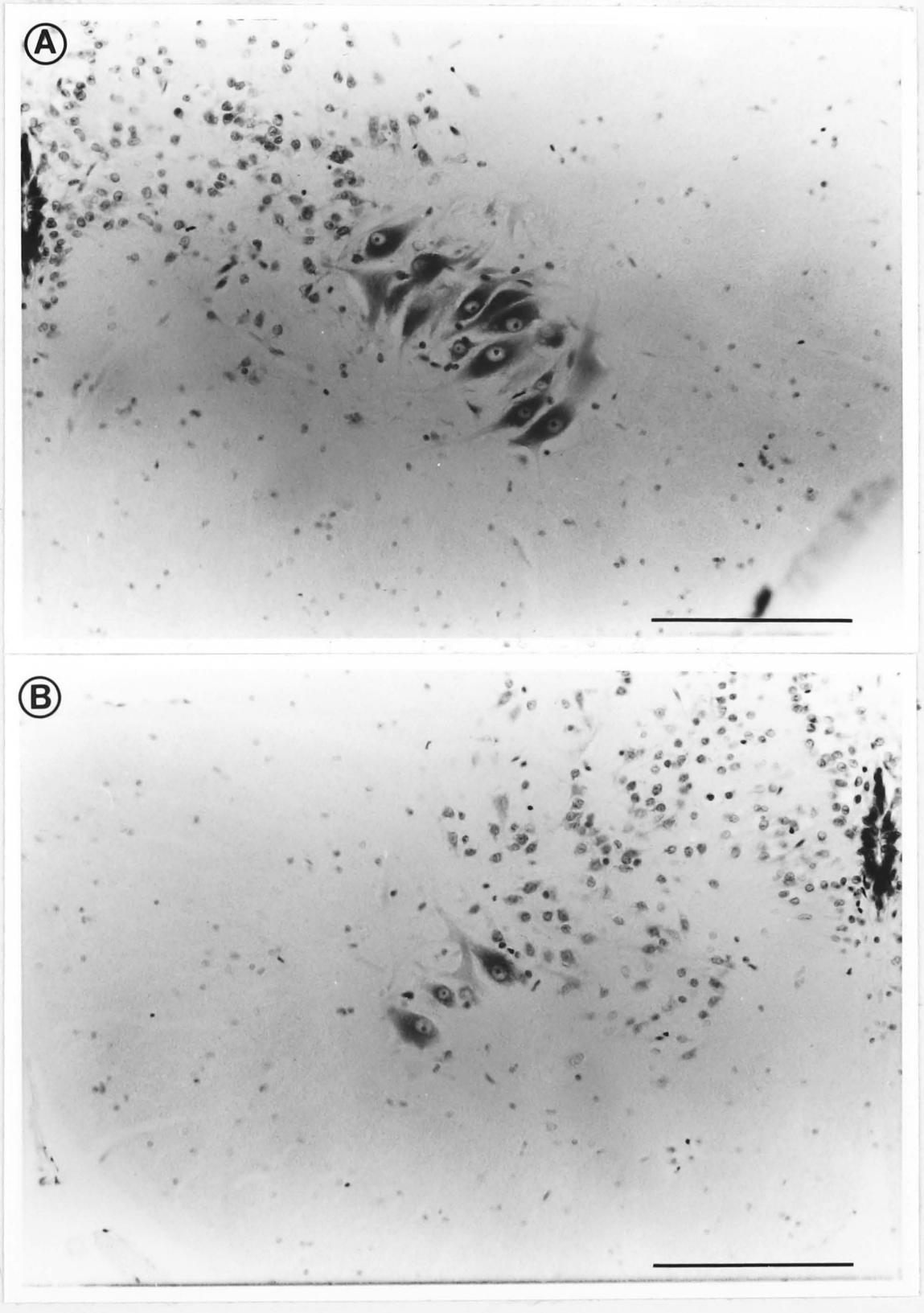
(a) Normal motoneurons

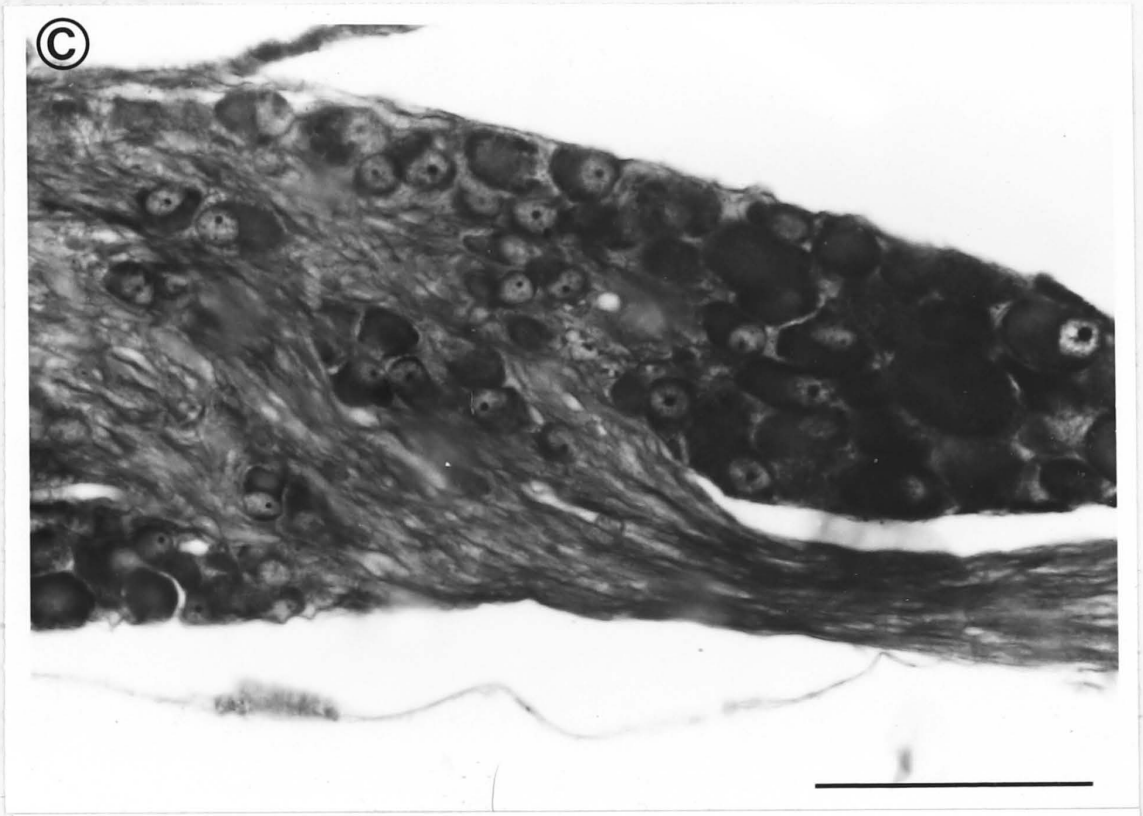
(b) Motoneurons on operated side

(c) Normal DRG 9

(d) DRG 9 on operated side

Bars = 100μm.


A
B


C

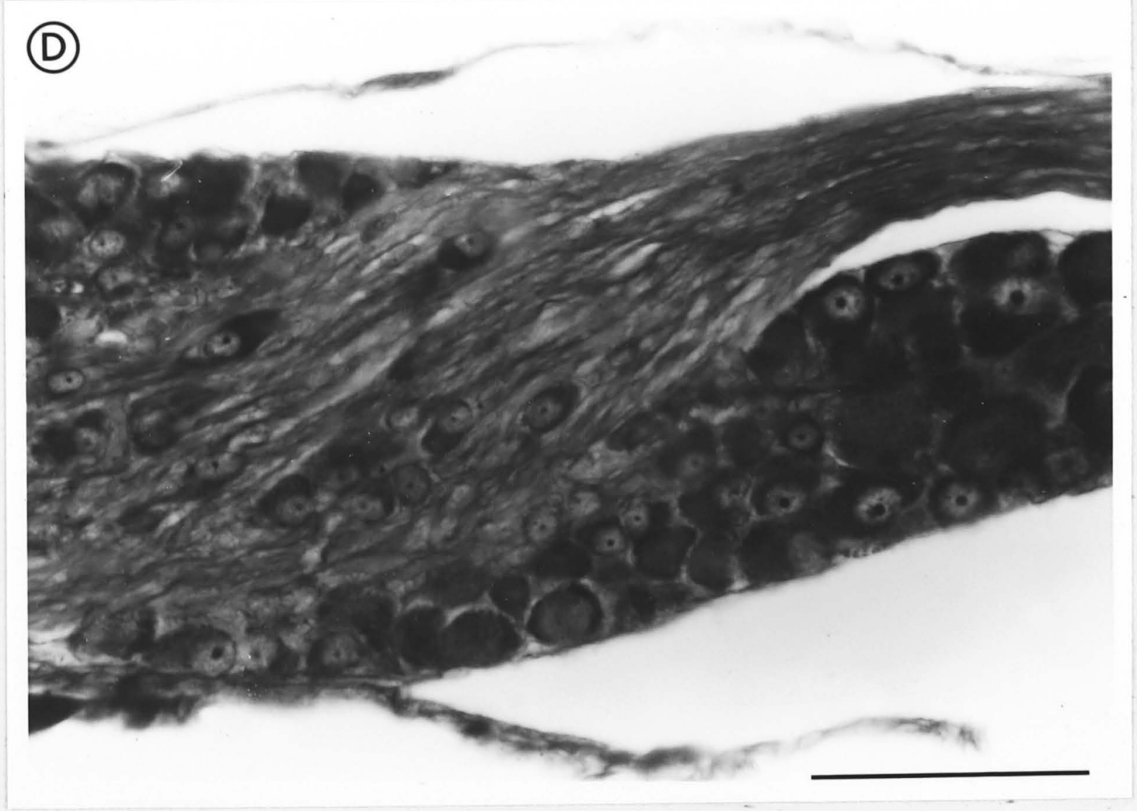
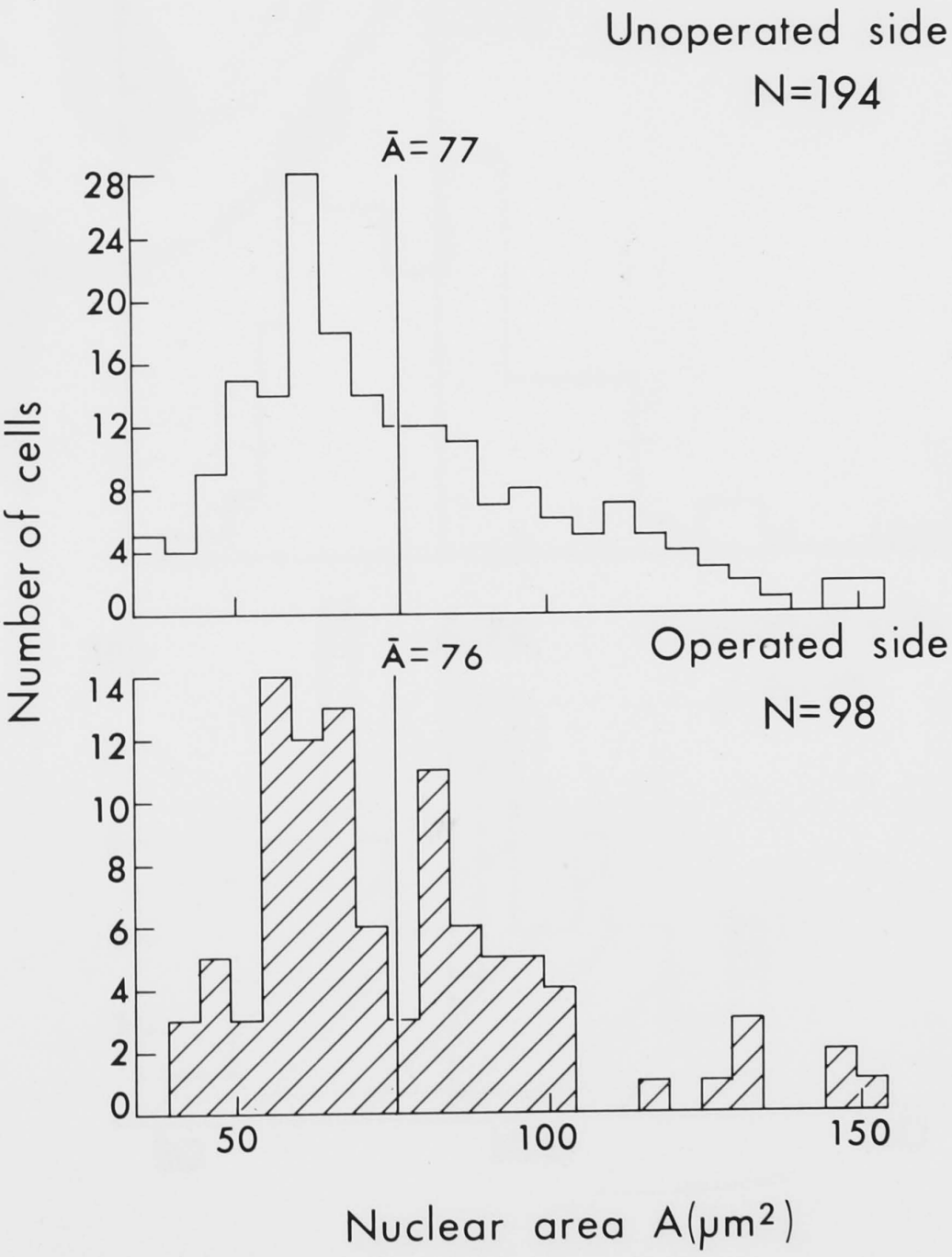
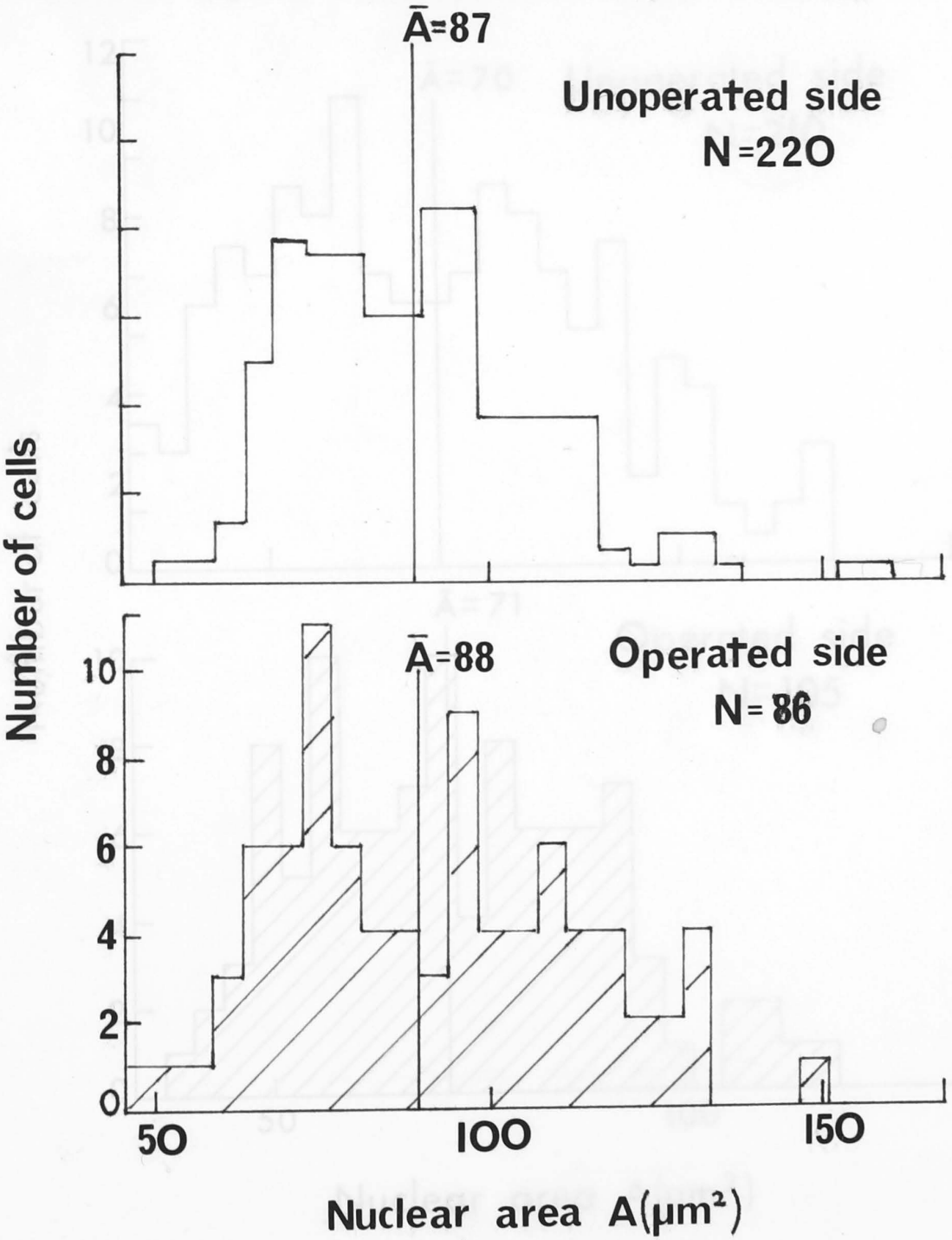

D

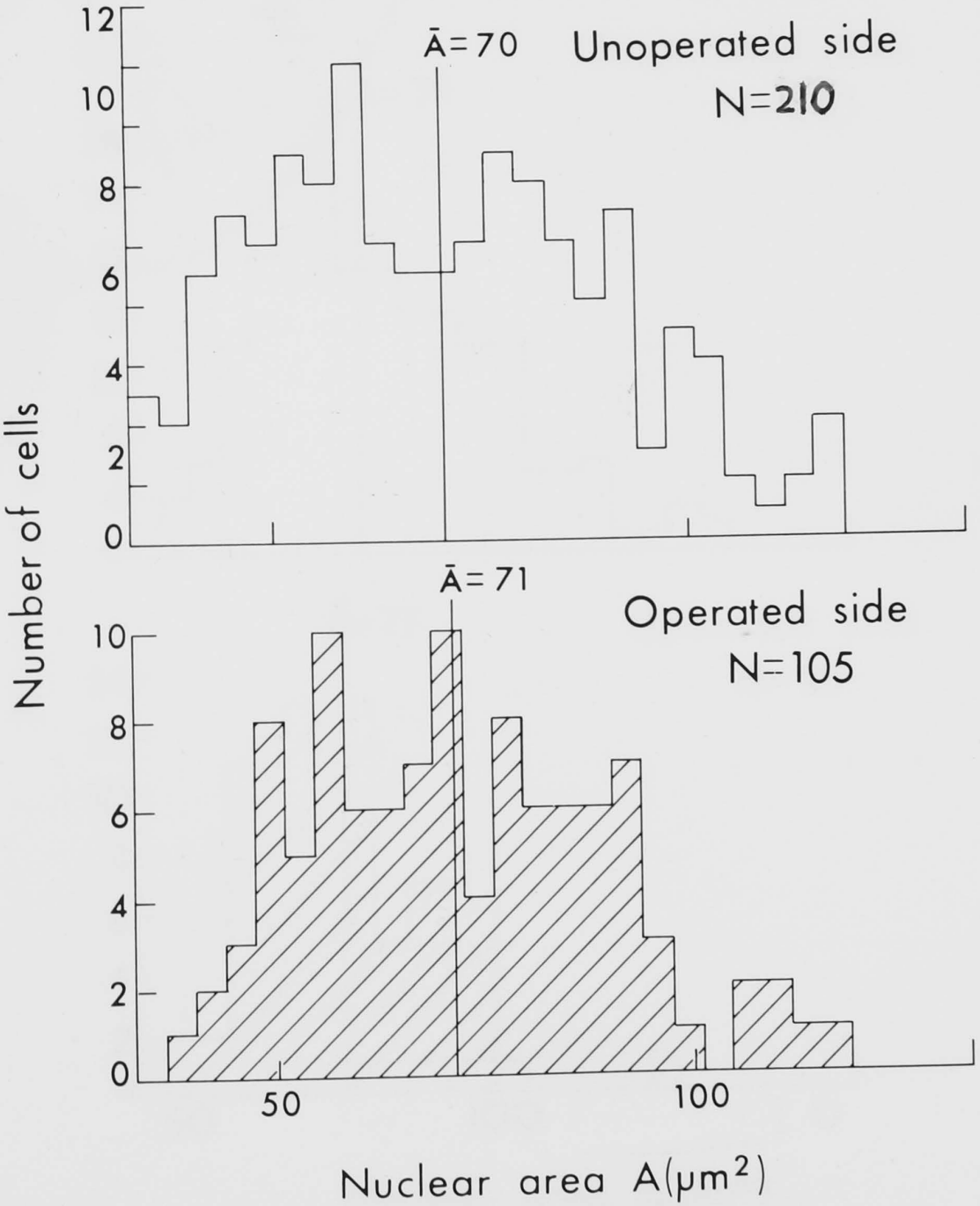
FIGURE 3.6 NUCLEAR AREAS OF LUMBAR MOTONEURONS SUPPLYING THE NORMAL HINDLIMB, AND TRANSPLANTED FORELIMB ANIMALS A - J

MOTONEURON NUCLEAR AREAS ANIMAL A

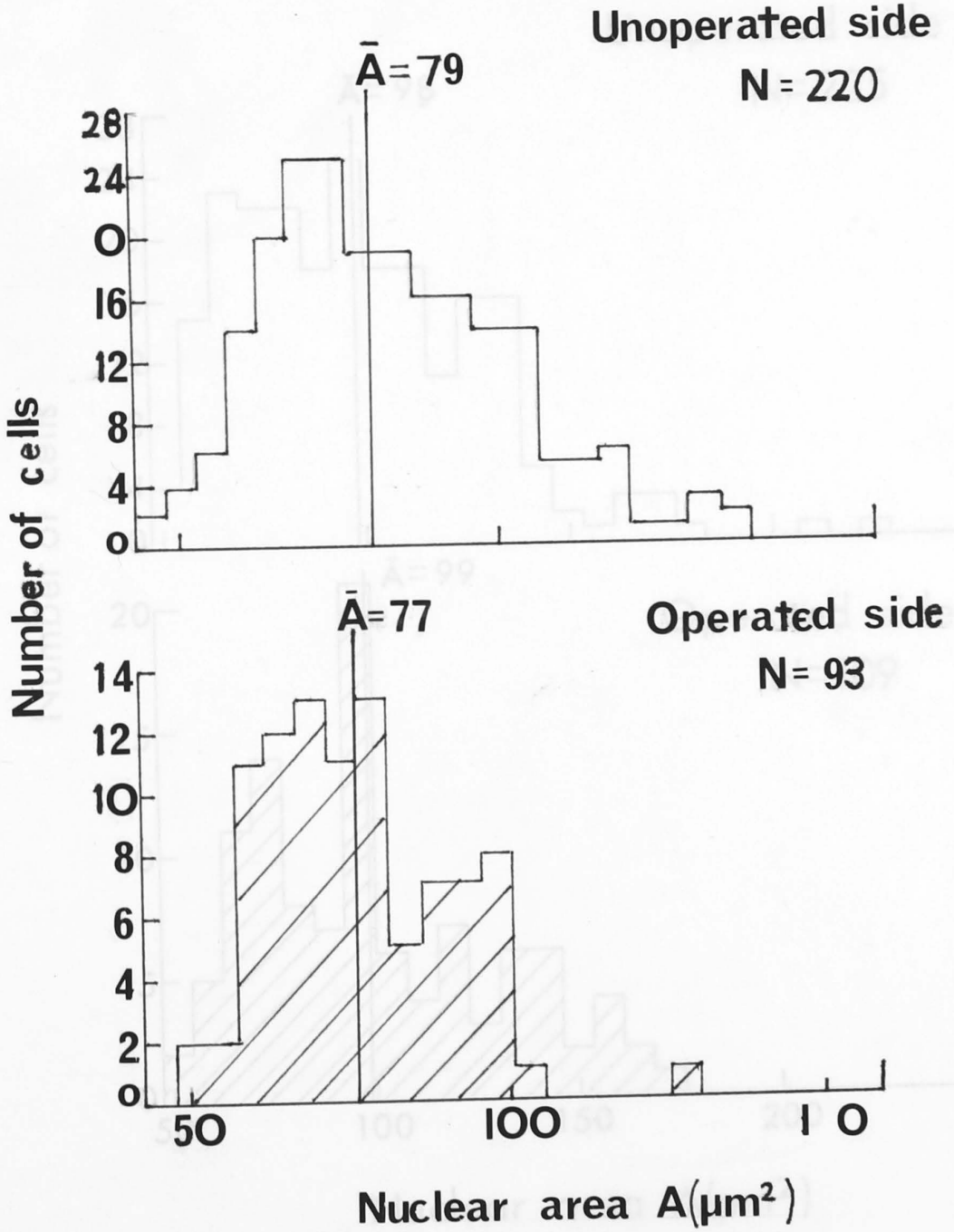



MOTONEURON NUCLEAR AREAS ANIMAL B
Ā=87
Unoperated side
N=220
Ā=88
Operated side
N=86
Number of cells
0
2
4
6
8
10
50
100
150
Nuclear area A(μm²)

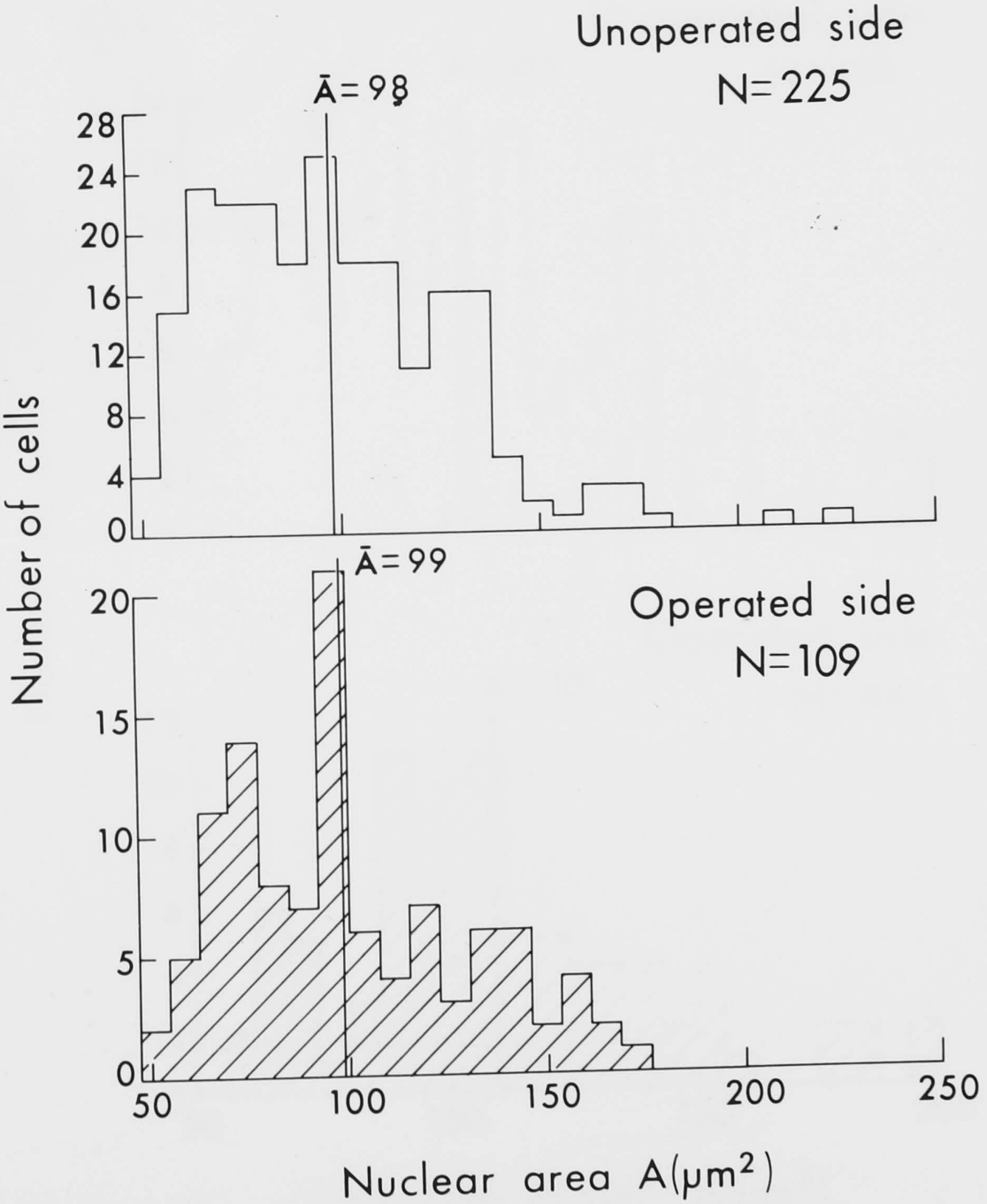
MOTONEURON NUCLEAR AREAS ANIMAL C



MOTONEURON NUCLEAR AREAS ANIMAL D



MOTONEURON NUCLEAR AREAS ANIMAL F



MOTONEURON NUCLEAR AREAS ANIMAL J

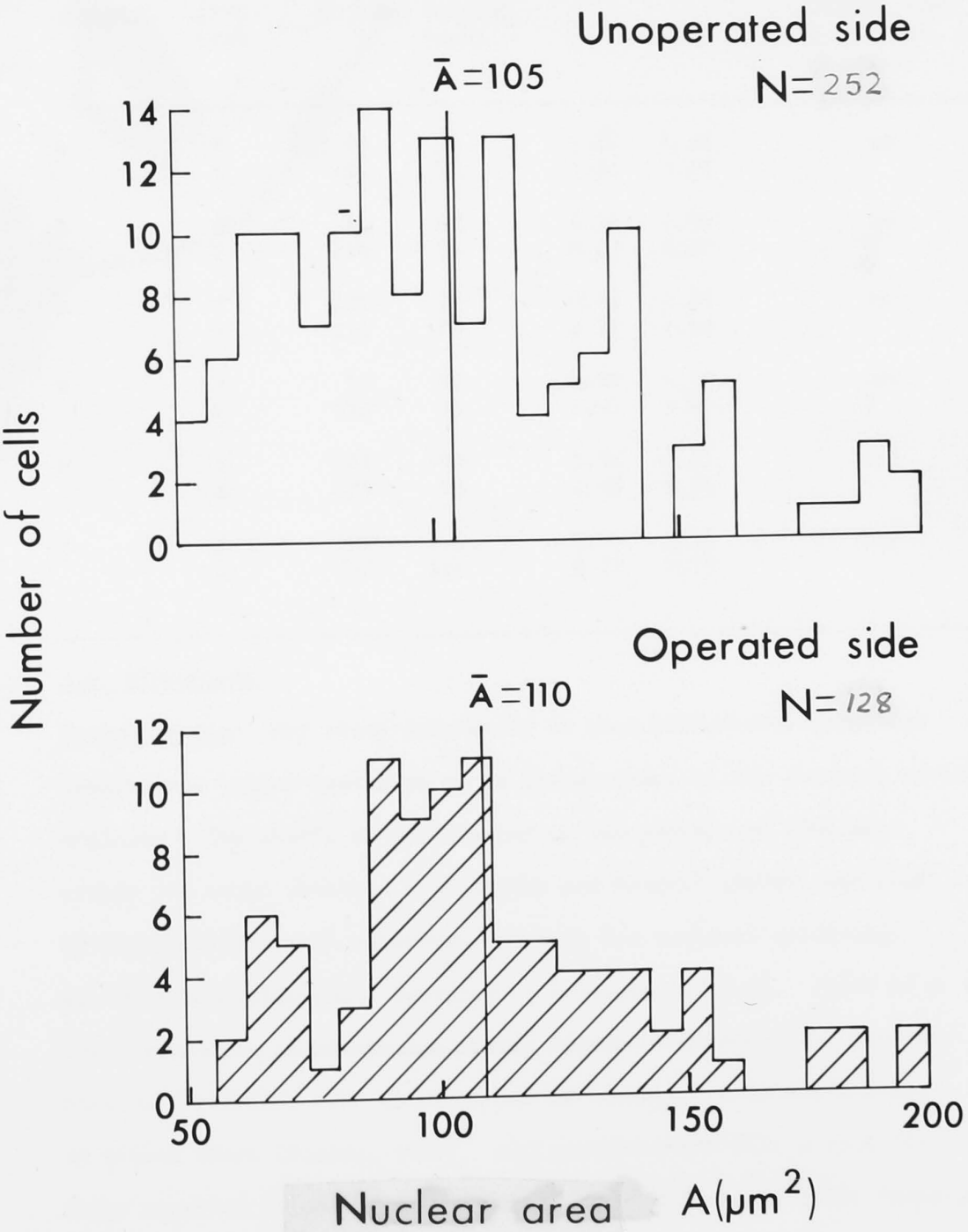


TABLE 3.4 SUMMARY : NUCLEAR AREA DISTRIBUTIONS OF LUMBAR MOTONEURONS SUPPLYING THE HINDLIMB (h) OR TRANSPLANTED FORELIMB (f).

ANIMAL	FORE-OR HIND-LIMB	No NEURONS	MEAN NUCLEAR (μm^2)	SKEW ($\delta 1$)	KURTOSIS ($\delta 2$)	KOLMOGOROV-SMIRNOV TEST (sig. diff. at 5% level)
A	f	89	76	1.20	4.36	no
	h	194	77	0.80	3.13	
B	f	86	88	0.36	2.38	no
	h	220	87	0.53	2.60	
C	f	105	71	0.31	2.28	no
	h	210	70	0.37	2.50	
D	f	93	77	0.99	4.10	no
	h	220	79	0.91	3.90	
F	f	109	99	0.56	2.45	no
	h	225	98	0.89	4.28	
J	f	128	110	0.76	3.38	yes
	h	252	106	0.72	3.15	

3.4 DISCUSSION

Neuron counts: Any study purporting to show differences in neuron populations raises questions as to the accuracy of the counting method employed. The counts of motoneurons on the unoperated side were within the range obtained by Hollyday and Mendell (1976), and also by Hughes (1961) when allowance was made for nucleoli splitting, but were less than those reported by Prestige (1967 a). There is a large variation in motoneuron totals between individuals of separate batches (Prestige, 1967 a) which may be accentuated by differences in growth rates (Hughes, 1961). DRG neuron totals were within the range reported in previous studies (Hughes and Tschumi, 1958; Prestige, 1965; Rubin and Mendell, 1980).

In the present study it was explicitly assumed that motoneurons on both sides of the cord displayed similar morphological characteristics, however the possibility that there existed a population of small, relatively undifferentiated motoneurons on the operated side of the cord could not be entirely excluded (see also below).

Motoneuron nuclear areas:

The mean nuclear areas were in general lower than those reported by Hollyday and Mendell (1976). The motoneurons increase in size with the growth of the animal, except in the stages approaching metamorphosis (Beaudoin, 1955; Pollack, 1969 a), and the majority of animals in the present study had just completed metamorphosis. The most mature animals in this study had motoneurons within the size range given by Hollyday and Mendell (1976).

In mammals the distribution of motoneurons supplying individual muscles is frequently bimodal (Burke, Strick, Kanda, Kim and Walmsley, 1977; McHanwell and Biscoe, 1981 b). It is likely that this division represents separate populations of gamma and alpha motoneurons, since the gamma motoneurons were significantly smaller in populations examined (Strick, Burke, Kanda, Kim and Walmsley, 1976) and may consist of over 20% of the total population, as judged by relative sizes (McHanwell and Biscoe, 1981 b). Nonetheless, a unimodal distribution was found in the facial nucleus of the rat (Martin, Caddy and Biscoe, 1977), possibly due to an absence of gamma motoneurons, or overlap of the distributions of alpha and gamma motoneurons.

In frogs there is no separate gamma motor system, since the intrafusal muscle fibres are supplied by collaterals of the extrafusal fibres (review by Barker, 1974). The extrafusal fibres can be subdivided into a homogeneous slow population, and two categories of a fast, or twitch population (Luff and Proske, 1979). The twitch motor system consists of large diameter (9-20μm) fibres whereas the slow fibres are less than 8μm in diameter (in Simpson, 1976) and it is likely that these size differences are reflected in terms of soma size. Although in the present study the data might indicate a heterogeneous neuron population, any attempt to discriminate subpopulations would require analysis at the level of individual motor nuclei. The observation that motoneurons located most laterally, particularly in segment 10, were in general smaller than their more medial neighbours, raises the possibility that there are also size variations between neurons supplying particular regions of the limb. The motoneurons in question supply the musculature of the foot (Lamb, 1976).

Although there was a reduction in the number of lumbar motoneurons supplying the transplanted forelimbs, there was not a consistent reduction in their nuclear areas. Several authors have reported a significant difference in nuclear areas of motoneurons following changes in the size of the peripheral field. Pollack (1969 b) found no significant difference between the *mean* nuclear areas of motoneurons supplying one, or three forelimbs in *Rana pipiens*, but there was a significant increase in the *numbers* of large neurons on the hyperplastic side. Hollyday and Mendell (1976) found both a significant increase in the mean nuclear areas

and proportions of large neurons supplying supernumerary hindlimbs of *Xenopus*. The increases of neuron size were found in frogs where the extra hindlimb had been transplanted at relatively late stages of development.

Competition and/or incompatibility as causes of neuron death?

In the present study it has been shown that lumbar motoneurons survive in reduced numbers, following innervation of a transplanted forelimb from the outset. This would tend to rule out incompatibility between motoneurons and limb muscles as a direct cause of lumbar motoneuron death, however it would still be possible that particular motoneurons must connect with specific limb regions i.e. the homologous regions of the transplanted forelimb (This question is addressed in Chapter 4). By the addition of a limb (Morris, 1978; Rubin and Mendell, 1980), or the reversal or translocation of several cord segments (Lance-Jones and Landmesser, 1981 b), or rotation of a limb bud prior to axon outgrowth (Stirling and Summerbell, 1979; Summerbell and Stirling, 1981), motoneurons can be made to innervate atypical limb regions, yet they survive. In those situations the surviving neurons would probably not have been required to compete with other neurons. If, on the other hand, all the motoneurons are forced to innervate only part of a limb, by removal of one or more limb segments, the atypically projecting neurons die (Lamb, 1981 b). Although it is not known whether the motoneurons form connections within the limb in this situation, it does suggest that competition plays a part in their removal.

Direct incompatibility has been indicated by other experiments of Lamb (1979). In adult *Xenopus* the caudal motoneurons never project to the proximal limb, but do so during development (Lamb, 1976). These neurons are eliminated by neuron death early in development (Lamb, 1977). Removal of the rostral segments of the cord, however, does not prevent their death (Lamb, 1979). In the present study, incompatibility of lumbar motoneurons and myotome muscle fibres appeared to be the most likely cause of neuron death. This extends the observations of Letinsky (1974), who replaced the hindlimb of *Rana catesbeiana* with three myotomes, containing 200-300 muscle fibres, up to stages IX to X (equivalent to Nieuwkoop and Faber stages 52-53). He presented electrophysiological evidence for innervation of the myotome fibres by the limb motoneurons, and noted that the motoneurons on the operated side were retarded in their differentiation, towards the onset of the period of neuron death. In the complementary operation, where motoneurons at the thoracic levels are forced to innervate a transplanted limb, the motoneurons form transmitting junctions (Morris, 1978) but fail to provide trophic support to the muscles (Harrison, 1907; Detwiler, 1920 a; Miner, 1956; Székely and Szentágothai, 1962; Hollyday and Mendell, 1975; Morris, 1978).

The operation of replacement of the forelimb with a hindlimb was successful, but the hindlimbs appeared atrophic. A further investigation of this result, in terms of the fate of the motoneurons, is warranted. Similarly Weiss (1924, reported in Hughes, 1964 a) also found that it was much more difficult to obtain functional innervation of hindlimbs grafted near the forelimb, whereas the converse

manipulation was often successful (see also Hughes, 1964 a). Brachial motoneurons may be less compatible with hindlimb muscles than lumbar motoneurons are with forelimb muscles. It is known that the lumbar motoneurons of the frog receive relatively unspecific projections from muscle sensory afferents (Simpson, 1976) in contrast to the brachial motoneurons, which parallels the situation in the cat (Frank and Westerfield, 1982 a) and this difference may be reflected in the periphery. Alternatively the brachial motoneurons may be unable to support such a large peripheral field in relation to their normal target.

Comments regarding the lumbar DRG neurons are limited by the lack of knowledge of their central projections (see Chapter 4 concerning reflex behaviour). The composition of the DRG may be predicted to some extent in terms of neuron sizes. Over half the afferent fibres from the frog DRG are small and unmyelinated (Baker and Richter, 1977), and most likely mediate pain responses, whereas the large diameter fibres mediating monosynaptic connections onto brachial motoneurons (Frank and Westerfield, 1982 a), and long ascending pathways are only a small component of the total. In the present study both small and large diameter neurons were observed. Again, the survival of a substantial number of DRG neurons weighs against incompatibility as a cause of death, however the comments concerning specificity of lumbar as opposed to brachial motoneurons also apply here.

In summary, incompatibility, in terms of neuron death and muscle atrophy, can be shown in experimental mismatches, but even in the most extreme mismatches the motoneurons form transmitting junctions. Within the homotypic limb, competition may be then essential for the removal of connections.

Reduction in neuron numbers:

The normal forelimb is supplied by about 1100 motoneurons (Fortune and Blackler, 1976), slightly more than reports for the hindlimb, and the neurons are of a similar size range. There are a number of factors which may have contributed to the reduction in lumbar neuron totals following replacement of the hindlimb with a forelimb. The trivial explanation that one or more segmental nerves failed to reach the limb was ruled out in Chapter 2. It also seems unlikely that the operation *per se* was a major contributing factor to the reduction, since a regenerated hindlimb supported the normal number of motoneurons. Motor axins first enter the limb bud at stage 50 (Lamb, 1974), after the time of the operation, and at least some can regenerate their axons at these stages (Lamb, 1981 c).

There may be some accentuated incompatibility in this experimental situation as discussed above. For example, Hughes (1964 a, b) transplanted a forelimb alongside the hindlimb in *Eleutherodactylus*. The limb was supplied by collaterals from lumbar nerve fibres (see Stephenson, 1979) and became mobile, however these collaterals were withdrawn towards hatching. The lumbar motoneurons therefore selectively innervated hindlimb, as opposed to forelimb, muscles.

Motoneurons are generally not very responsive to changes in the size of the peripheral field. A supernumerary limb increases totals by only as much as 28% in *Xenopus* (Hollyday and Mendell, 1976), and a single limb may be forced to support twice the normal total (Lamb, 1980). The forelimb in the adult is only about 15% of the mass of the hindlimb*, and such a large reduction might be responsible for the enhancement of neuron death. The number of primary myotubes at the onset of innervation is not known, however.

Another possibility is that the slower differentiation of fore- as opposed to hindlimbs is disadvantageous to lumbar motoneurons. It is conspicuous in both frogs and chickens that the time course of motoneuron death is protracted in the brachial as opposed to lumbar cord (frog - Pollack, 1969 a; chicken - Hamburger, 1975; Oppenheim and Majors-Willard, 1978). This difference might also be significant in terms of the detection of transient diffuse projections early in development of the chick (see Lance-Jones and Landmesser, 1981 b).

*see Table opposite page 51.

3.5 SUMMARY

1. The hindlimb bud of stage 49 tadpoles of *Xenopus laevis* was replaced with a stage 50 forelimb bud, or several myotomes. Some forelimb buds were also replaced with a hindlimb bud.

2. The mytomes failed to grow, became atrophic and disappeared prior to stage 58. The transplanted forelimbs completely differentiated and received motor and sensory innervation from the ipsilateral lumbar cord. The transplanted hindlimbs were atrophic. The limb motoneurons were identified by morphological criteria.

3. The number of motoneurons surviving replacement of the hindlimb with myotomes was not greater than that surviving unilateral limb amputation. The transplanted forelimb supported between 39% to 55% (mean 46%) as many motoneurons as the contralateral hindlimb, and the LMC on the operated side was of normal length.

4. The size distributions of lumbar motoneurons supplying the hindlimb, or transplanted forelimb were positively skewed in 13 out of 14 LMC's, and leptokurtic in all 14. The size distributions of motoneurons on the unoperated as opposed to operated sides were significanly different (Kolmogorov-Smirnov test) in 2 out of 7 cases.

5. It was concluded that some lumbar motor and sensory neurons attained normal levels of maturity in terms of nuclear size, after innervating a forelimb, but not myotomes, from the onset.

CHAPTER FOUR

THE PROJECTION FROM THE LUMBAR SPINAL CORD TO A TRANSPLANTED FORELIMB IN *XENOPUS*

III MOTOR AND REFLEX BEHAVIOUR OF THE LIMB

4.1 INTRODUCTION

The co-ordinated activation of muscles is dictated by the firing patterns of their motoneurons, which in turn is governed by impulses received from central interneurons. It is not yet clear how the correspondence between the central and peripheral connections of the motoneurons arises during development.

Some of the earlier hypotheses concerning this problem arose from the observations by Detwiler (1920 a) in the urodele amphibia. A transplanted forelimb rudiment that received innervation from the brachial plexus developed movements in co-ordination with the adjacent limb. Weiss (1922, 1937 a, b, c, d) repeated this experiment, using a differentiated limb, and also found that the limb developed co-ordinated, or 'homologous' movements. It was proposed that upon reinnervation of different muscles, the functional relations (Weiss, 1926), or central connections (Sperry, 1941) of motoneurons could make appropriate rearrangements, and this peripheral specification of the motoneurons occurred also during development. Most efforts to show changes in central connections onto motoneurons following peripheral nerve crosses in neonate mammals, failed however (Sperry, 1941, 1945 a; Weiss and Hoag, 1946). The lack of central adaptability placed the burden of functional recovery on events in the periphery, and there is now substantial evidence for selective reinnervation of muscle territories as the basis for recovery of co-ordination in axolotl limbs, upon regeneration of mixed motor nerves (Grimm, 1971; Cass, Sutton and Mark, 1973; Cass and Mark, 1975; Bennett and Raftos, 1977; Stephenson, 1979).

Central connections form on the limb motoneurons in a retrograde sequence during development. In the chicken at about the 5th day of development neuromuscular junctions have formed in the limb (Landmesser and Morris, 1975) and the motoneurons receive the first synapses from presumptive commissural and association interneurons (Oppenheim, Chu-Wang and Foelix, 1975). On the 7th day, flexor and extensor muscles can be shown to be activated in alternation during periods of spontaneous mobility (Bekoff, et al 1975). The interneurons in turn receive afferent terminals, to complete the first cutaneous reflex arcs on days 7 - 8, and at days 9 - 12 the first proprioceptive reflexes have formed (Windle and Orr, 1934). A similar developmental sequence has been described largely in behavioural terms in the anuran tadpole by Hughes and Prestige (1967). There then exists the possibility that the periphery exerts some selective influence on the acquisition of central connections by motoneurons during development (Landmesser, 1980).

Peripheral specification of neuron connections has also been raised with respect to the sensory ganglia, both during reinnervation and development. Weiss (1942) transplanted an eye to the region of the nose or ear in urodeles, at mid-larval stages. After metamorphosis a cutaneous stimulus to the transplant eye could elicit a blink reflex in the normal eye, a response normally mediated by the ophthalmic division of the trigeminal nerve. It was proposed that the eye had appropriately modified the central relations of the sensory neurons that by chance reinnervated it. Although this result was challeneged (Székely, 1959 b), other experiments concerning

wiping reflexes elicited from translocated skin in anurans (Miner, 1956; Baker, 1968; Jacobson and Baker, 1969; Baker, Corner and Veltman, 1978; Székely, Matesz, Baker and Antal, 1982), withdrawal reflexes from supernumerary limbs (Miner, 1956; Hollyday and Mendell, 1975), head withdrawal reflexes following cross-anastomosis of cranial nerves (Griersmith and Mark, 1982) and reflex connections formed by foreign sensory neurons onto brachial motoneurons (Frank and Westerfield, 1982) have tended to support the original proposition by Weiss.

In this study the motor and reflex behaviour of a transplanted forelimb, innervated from the onset by lumbar motoneurons, was examined in behavioural and anatomical terms. Peripheral specification of sensory and motor connections was tested by observations of reflex and motor coordination of the transplanted limb.

4.2 METHODS

Embryology: Forelimb buds were transplanted as described in the Methods Section of Chapter 2.

Behaviour: Natural terrestial and aquatic locomotion was recorded on Eastman Ektachrome 16 mm Video News Film, using a Bolex H16 EL cine camera at a speed of 50 frames per second. At this film speed each frame was exposed for about 7 milliseconds, and there was an interval between frames of about 13 milliseconds.

Reflexes were tested in post-metamorphic frogs at a room temperature of 17 - 21°C. Wiping reflexes were elicited by mechanical and/or chemical stimuli. The mechanical stimuli were repeated strokes with a 0.2 mm diameter nylon bristle mounted on wooden dowel (maximum force 1.2g). Chemical stimulation was by a single delivery of 2 - 3µl of 100% ethanol, or 0.5 M acetic acid from a standard Eppendorf tip onto a patch of skin of area less than 40 mm^2. The animal was immersed in water for at least a minute between tests. Limb withdrawal reflexes were elicited by a light touch to the skin with the conical point of a glass rod. At least a minute expired between repetition of each stimulus. To prevent swimming after touching the hindlimbs it was first necessary to transect the spinal cord at the level of the medulla, under MS 222 anaesthesia, and allow the animal time to completely recover from the effects.

Histology: Motoneurons supplying one of 3 regions of the transplanted forelimbs were located in a total of 9 animals. The limb regions selected were the superficial extensors and flexors of the arm (M. anconeus and M. sternoradialis) and extensors of the hand (M. extensores digitorum communis longus and breves superficiales). The animals had reached developmental stages 58 - 60, when lumbar motoneuron death had largely ceased (Hughes, 1961; Prestige, 1967), and the skin of the limb was translucent. Transplanted forelimbs participated in swimming movements, and individual muscle fibres could be seen at 50 X under the dissecting microscope.

Injections: Following anaesthesia in MS 222 solution, each animal was placed on one side and kept moist. A small skin incision was made with tungsten needles through which an injection of 30% horseradish peroxidase (HRP - Boehringer Mannheim grad. 1) in 0.65% saline was delivered via a broken glass microelectrode mounted on a micromanipulator. The amount delivered (approximately 0.001µl) was controlled manually by varying air pressure in the electrode with an attached 100 ml syringe and polythylene tubing. The extent of the injection could be judged by the brown discolouration, which disappeared within 30 seconds without spreading. Uptake of HRP is largely by endocytosis from growing axon tips or presynaptic terminals at the neuromuscular junctions (Chu-Wang and Oppenheim, 1980 a). Since only the superficial musculature was injected, motor axons passing to other limb regions could not have been severed.

Histochemistry: The procedure followed that of Lamb (1977). The wax embedding procedure permits sections of optimum thickness for the density of motoneurons encountered. An immersion time of 1 hour in diaminobenzidine tetrahydrochloride (DAB) was sufficient when spinal cords were completely free of surrounding tissue. Serial sections were cut at 10µm thickness, and every 10th section was counterstained with toluidine blue. Sections were dehydrated and cleared as rapidly as possible, as the reagents gradually degraded the HRP reaction product.

4.3 RESULTS

Motor Co-ordination of a Forelimb in the Hindlimb Position:

All transplanted forelimbs received innervation from the lumbar segments (8, 9 and 10), and were motile and sensitive to cutaneous stimuli (Fig. 4.1 (a). The first movements of the transplant forelimb were evident at Nieuwkoop and Faber stages 55 - 56, 1 or 2 stages after the first hindlimb movements. The forelimbs are normally enclosed in opercular pouches, from which they emerge at about stage 58. Movements are first evident 1 or 2 stages prior to emergence. The first transplant limb movements were difficult to observe due to their rapidity, and to the position and shortness of the limbs. However the 'flare' movement, a bilateral flexion of the limbs which drew them outwards from the body immediately after a bout of swimming (Hughes and Prestige, 1967), was seen in the transplanted forelimbs at the early stages of motility.

At the onset of metamorphosis the swimming movements of the hindlimbs supplanted those of the tail as it was resorbed. The hindlimb swimming movements consisted of a rapid, synchronous extension, comprising the power stroke, followed by limb flexion. The hindlimb extension was often, but not always accompanied by a stroke of one or both forelimbs. Co-ordination of the hindlimbs during swimming is dependent on intact labyrinths. Rapid extension of the hindlimb of operated animals was always accompanied by extension of the transplanted forelimb (Fig. 4.1 a), followed by limb flexion. In normal animals at low speeds the limb coupling loses its precision,

and on a moist surface there is a tendency for the limbs to move in alternation, which was also observed in operated animals (Figs. 4.1 b, c). At all speeds instances could be found where the forelimbs were not used, or were used in opposite phase to the hindlimb, during a power stroke of the transplanted limb.

The orientation of the transplanted limbs had no bearing on their co-ordinated flexion and extension, even when the movements could not aid forward progression. In one operated animal of the 20 filmed (animal H), the transplanted forelimb was deficient in the extent of movement of the elbow and hand. This limb was well-formed, with all the bones of the normal left pectoral girdle, however the innervation from spinal segment 10, which normally supplies the distal hindlimb (Lamb, 1976), was sparse (see Fig. 3.3).

Motor somatotopy: The basic pattern of hindlimb co-ordination evident in transplanted forelimbs raised the possibility that they were selectively innervated by the lumbar motoneurons. This was investigated in terms of the somatotopic organization of the motor projection to the limb. Horseradish peroxidase (HRP) was injected to 1 of 3 limb regions in a total of 9 animals with well-formed limbs. The motoneuron somas supplying those regions were subsequently located within the spinal cord (Fig. 4.2 a). The limb regions selected were the superficial extensors and flexors of the arm (M. anconeus, and M. sternoradialis) and extensors of the hand (M. extensores digitorum communis longus and breves superficiales).

FIGURE 4.1 MOTOR CO-ORDINATION OF A FORELIMB IN THE HINDLIMB POSITION

(a) Response to touch

(b) Swimming at low speed

(c) Stepping on a moist surface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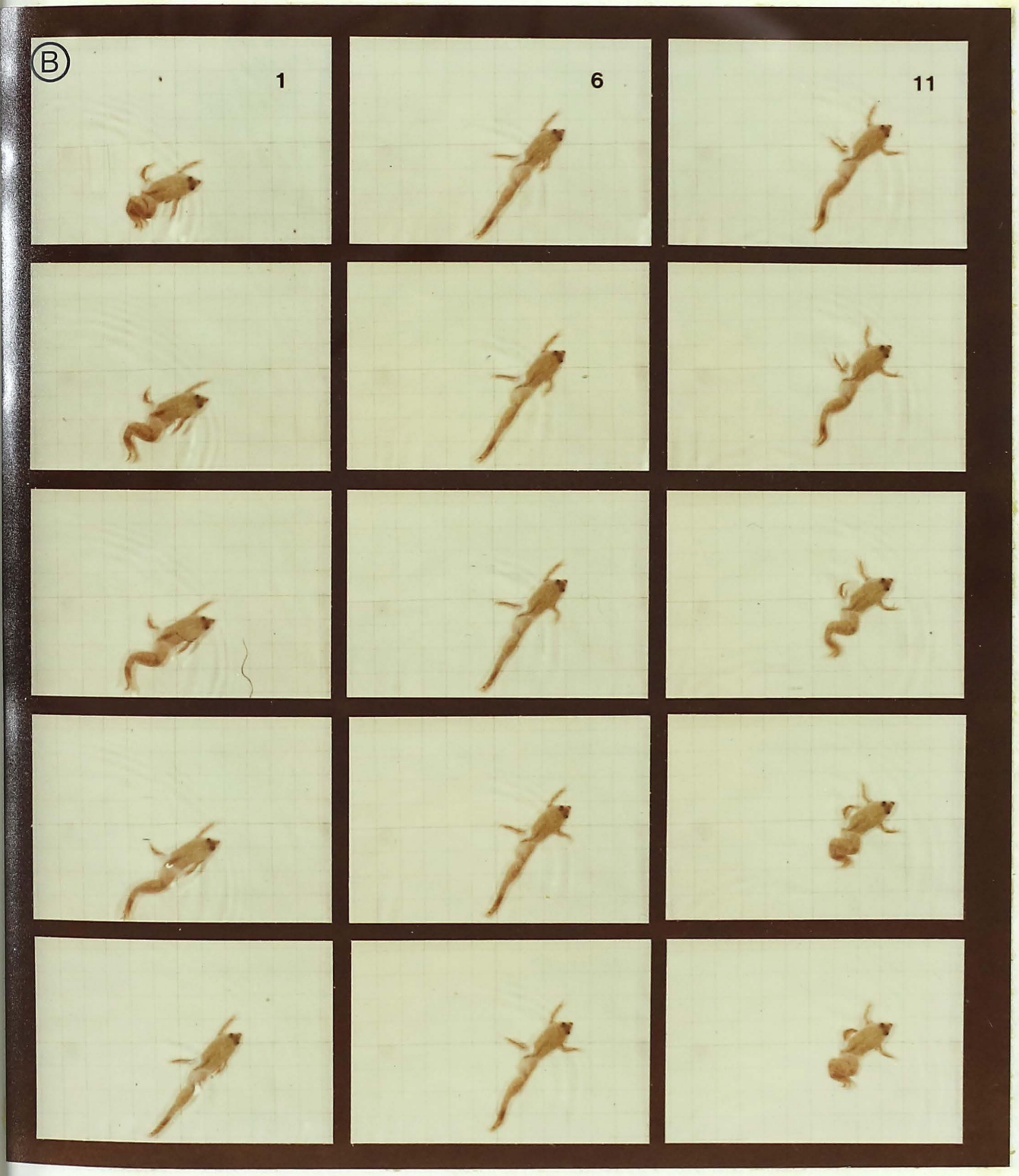
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The lateral motor column (LMC) on the operated side was of normal length and contained about half as many motoneurons (~500) as the operated side, at stages 58 - 60. The period of motoneuron death had almost concluded at those stages (Hughes, 1961; Prestige, 1967 a). HRP labelled cells were only found in the LMC on the operated side, generally amongst other unlabelled motoneurons. The centre of the distribution of labelled cells along the rostrocaudal axis varied according to the limb region injected, but not according to the limb orientation. Motoneurons supplying the extensor musculature of the hand were located towards the caudal end of the LMC, regardless of whether the extensor surface faced rostrally, as in the normal hindlimb, or caudally. Most motoneurons supplying the flexor musculature of the elbow were clustered more rostrally. Since the locations of labelled motoneurons did not vary with limb orientation, and the total number was quite variable from injections of similar volume and appearance, the results for each group of 3 animals were pooled (Fig. 4.2 b).

In the most rostral region of the LMC only a few motoneurons were found per section on the operated side, however there was generally an increase of numbers per section in segment 10. When the LMC was divided into four equal regions at this level, a tendency for the labelled motoneurons supplying extensors of the hand to be clustered more laterally within the LMC was evident (Fig. 4.2 b).

FIGURE 4.2 a HRP LABELLED MOTONEURONS SUPPLYING A TRANSPLANTED FORELIMB

The soma and main dendrites contain a brown granular reaction product. 10μm thick section, toluidine blue counterstain. Bar: 100μm

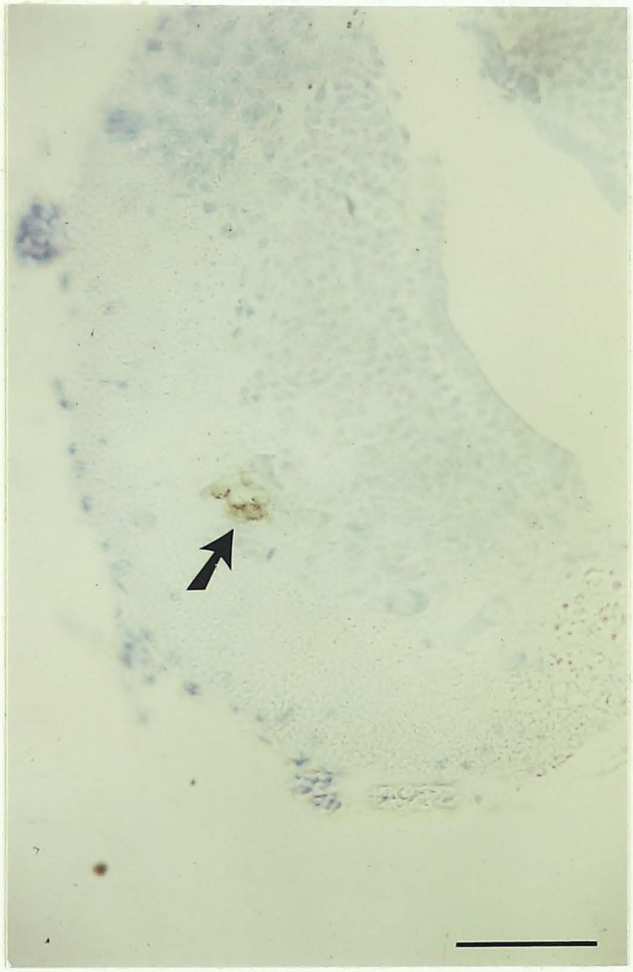
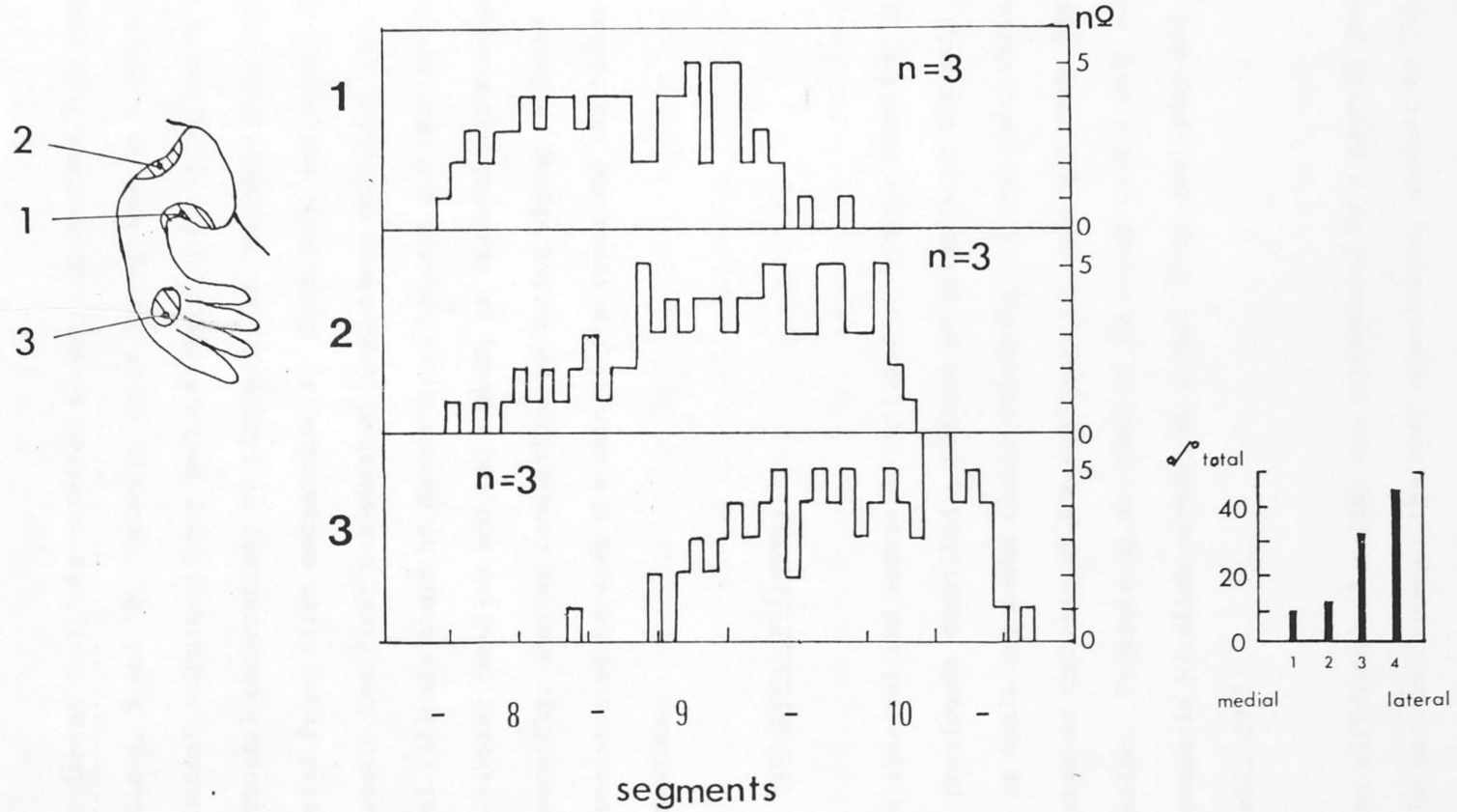


FIGURE 4.2 b ROSTROCAUDAL AND MEDIOLATERAL DISTRIBUTION OF LABELLED LUMBAR MOTONEURONS PROJECTING TO MUSCLES OF A TRANSPLANTED FORELIMB

Data was pooled for each group of 3 animals. The location of the spinal cord segments was estimated from the midpoints between the zones of entry of the dorsal roots. The mediolateral distribution of motoneurons supplying the extensors of the hand (region 3) was determined by dividing the lateral motor column into four equal sections at each level, and expressing the number of labelled motoneurons in each sector as a percentage of the total.

ROSTROCAUDAL AND MEDIOLATERAL DISTRIBUTION OF LUMBAR MOTONEURONS PROJECTING TO MUSCLES OF A TRANSPLANTED FORELIMB



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Reflex Behaviour of Forelimbs Innervated by Lumbar Segments:

Transplanted forelimbs received sensory innervation from lumbar ganglia 8, 9 and 10. Swimming could be provoked by a strong mechanical stimulus to any part of the skin of transplanted limbs. Behavioural observations of simple reflex responses were made in operated frogs after metamorphosis. There were two points of interest. The first was whether connections mediating limb withdrawal reflexes could be demonstrated between the skin of the transplanted forelimb and the lumbar, or brachial motoneurons, and secondly, whether connections mediating wiping reflexes could be demonstrated between the skin of the trunk and the lumbar motoneurons.

(i) Withdrawal reflexes:

The withdrawal and extension reflexes of *Xenopus* hindlimb have been previously described. Natural stimulation of the skin of the foot or ankle may evoke flexor withdrawal of the limb, while pressure on the skin of the calf near the knee may evoke limb extension. Pressure on the skin of the trunk evokes only small responses in hindlimb nerves, in spinal frogs (Hollyday and Mendell, 1975).

Reflex withdrawal of a limb was accompanied by a general increase in the postural tone of the body musculature, similar to the startle response evoked by vibrations in the water, in intact animals.

Stimulation of any part of the skin of the forelimb evoked limb flexion, largely at the shoulder joint. Reflex withdrawal of the forelimb was not accompanied by withdrawal of the transplanted forelimb. The threshold stimulus required to elicit swimming was higher for transplanted forelimbs than for the other limbs. Stimuli sufficient to evoke hindlimb or forelimb reflexes when applied to transplanted forelimbs elicited a small flexion response, which resembled the startle response of normal limbs, and was not accompanied by reflex responses in other limbs.

(ii) Wiping reflexes:

In agreement with Hollyday and Mendell (1975) wiping reflexes of the hindlimb could not be elicited by mechanical stimulation of the skin of the trunk. Mechanical stimulation of the skin of the head and shoulder, however, was sufficient to elicit a wiping reflex by the ipsilateral forelimb, accompanied by eye retraction, if in that vicinity. The forelimb ipsilateral to the stimulus was brushed forwards over the skin in one or more rapid strokes. Chemicals such as acetic acid or absolute ethanol were found to elicit either fore- or hindlimb wiping reflexes, and more readily in juveniles as opposed to large adults. Using 100% ethanol, which did not damage the skin, the wiping reflexogenous zones were plotted over the body surface (Fig. 4.3). Stimuli near the body midline resulted in one or both limbs responding. Simultaneous stimuli away from the midline in two zones often resulted in both limbs responding. The forelimb itself was wiped on the side of the head. The hindlimb was wiped with the heel of the contralateral hindlimb. The trunk

was wiped by the ipsilateral hindlimb, which was flexed so as to brace the foot over the site, and one or more scratches were then performed with the clawed toes of the foot. Wiping of the belly by the forelimb could not be elicited, possibly due to the absence of adductor muscles in the limb, which serve to support the body when sitting or after a jump, in other anuran species.

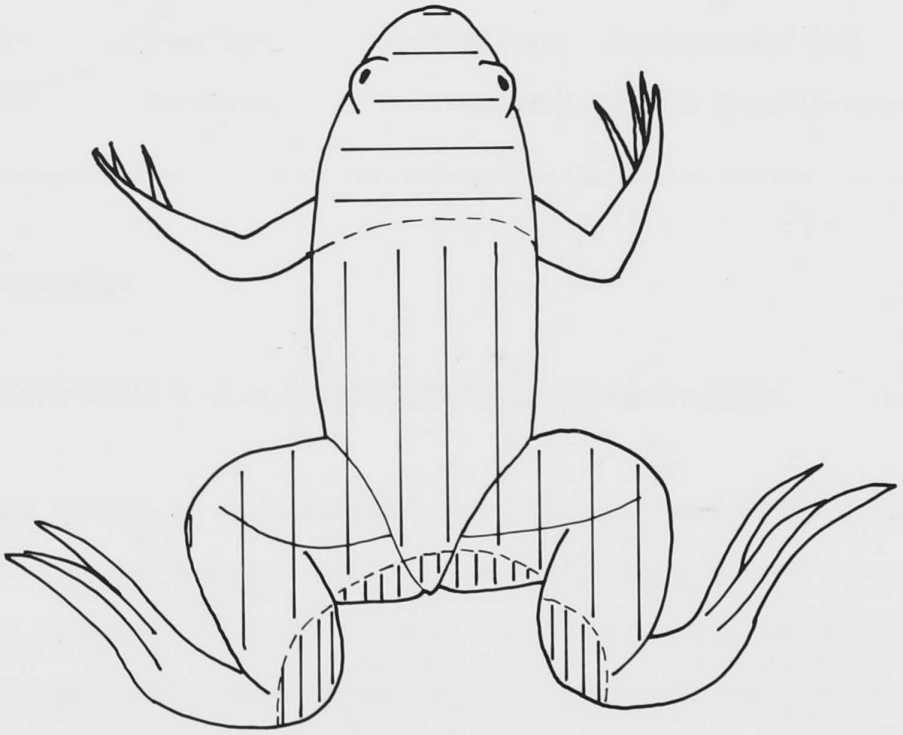
Ten operated animals had their limb and wiping reflexes tested after metamorphosis. In both operated and normal frogs there was considerable variability in the frequency of responses. Limb responses were most reliably evoked with stimuli applied at a rate of less than one per minute, whereas wiping responses were more easily elicited by rapid repetitive stimuli (Franzisket, 1963). Each form of stimulus was applied at least 10 times to each animal.

Mechanical stimuli which reliably evoked forelimb, but not hindlimb wiping reflexes, also failed to elicit movements in transplanted forelimbs, when applied in the ipsilateral hindlimb reflexogenous zone. Chemical stimuli which could elicit either forelimb or hindlimb wiping reflexes, elicited movements in transplanted forelimbs when applied in the ipsilateral hindlimb reflexogenous zone. Evoked movements were complicated and included limb flexion and rapid movements of the fingers. Contact with the trunk skin was generally not obtained, even where permitted by the particular orientation of the limb. These results are summarized below:

FIGURE 4.3 CUTANEOUS REFLEXOGENOUS ZONES IN *XENOPUS LAEVIS*

An irritating cutaneous stimulus prompts a wiping reflex by the limb indicated

CUTANEOUS REFLEXOGENOUS ZONES IN *XENOPUS LAEVIS*



arm

toe

heel

TABLE 4.1 WIPING REFLEXES IN *Xenopus laevis* (Refer to Fig 4.3 for location of reflexogenous zones)

Stimulus	Reflexogenous Zone	Reflex Response	
		Unoperated	Operated (transplanted limb)
Repetitive-mechanical	Hindlimb	None	None
	Forelimb	forelimb wipe	normal forelimb wipe
Noxious-chemical	Hindlimb	hindlimb wipe	transplanted limb
	Forelimb	forelimb wipe	normal forelimb wipe

4.4 DISCUSSION

Motor Co-ordination of a Forelimb in the Hindlimb Position:

The basic pattern of co-ordination of transplant forelimb movements with the hindlimb was in agreement with earlier observations in urodele amphibia (Székely, 1963) and chickens (Straznicky, 1963; Narayanan and Hamburger, 1971) showing that the basic locomotor output of the limb segments of the spinal cord is uninfluenced by the nature of the periphery. For example, substitution of lumbosacral with brachial segments in the 2½ day chick embryo gave rise to birds which, after hatching, could not move their legs in alternation, but abducted and adducted them simultaneously as though flapping wings (Narayanan and Hamburger, 1971).

The neural circuitry underlying co-ordinated movements of the limbs is not well understood. Studies in a wide range of vertebrates

have given rise to the concept of central 'pattern generators' for locomotion and other stereotyped motor behaviours, which may function in the absence of sensory and supraspinal inputs (for locomotion review see Grillner, 1975). For example, the limb motoneurons of frogs receive monosynaptic connections from primary afferent (Fukami, 1961; Frank and Westerfield, 1982 a), vestibular (Magherini, Precht and Richter, 1974) and probably reticular (Cruce, 1974 b) neurons. Motoneurons on opposite sides of the spinal cord at non-limb levels are also electrically coupled (Erulkar and Soller, 1980). The available evidence although somewhat contradictory, supports the view that these monosynaptic inputs may modulate, but do not comprise the pattern generator for limb movements, the basic circuitry of which consists of an interneuron network within the spinal cord (Székely and Czéh, 1976).

The swimming rhythm of the anuran tadpole, which consists of alternating contractions of myotomes on opposite sides of the body, also persists in the absence of sensory feedback via the lateral line system and dorsal roots (Stehower and Farel, 1980). Recently Kahn and Roberts (1982) proposed a model for the central pattern generator for swimming in *Xenopus* tadpoles, based on anatomical (Roberts and Clarke, 1982) and electrophysiological (Roberts and Kahn, 1982; Kahn and Roberts, 1982) evidence. They suggest that the contraction of the myotomes is achieved by autonomous rhythm generators, composed of interneurons on each side of the spinal cord, which drive the motoneurons under tonic excitation from the hindbrain. Alternation between the two sides at each level of the cord is then achieved by reciprocal inhibition between the rhythm generators. Under strong excitation

the motoneurons on each side of the cord may also fire in synchrony (Kahn and Roberts, 1982).

The 'primary' motoneurons which innervate the myotomes (Taylor, 1943; Hughes, 1959) at limb levels do not survive beyond metamorphosis (Székely, 1976). There is a possibility that the pattern generator for limb movements is interconnected, or a part of, the generator for swimming movements of the tail. This is supported by observations of animals where movements of the trunk play a major role during locomotion, as trunk convexity obviously accompanies limb protraction, and concavity, limb retraction (Brandle and Székely, 1973) during locomotion.

It should be clear from these considerations that although behavioural observations provide information concerning the patterns of motor output, the central neural basis of these motor patterns is a general problem that remains to be understood.

In the present study it was found that a limb reversed about the rostrocaudal axis displayed co-ordinated movements and motor somatotopy similar to a normal limb. Mendell and Hollyday (1976) noted co-ordinated movements in supernumerary limbs rotated so that the ventral limb surface faced dorsally, and Rubin and Mendell (1980) found normal somatotopic projections to several muscles of such limbs, in *Xenopus*. The outcome of limb rotation may differ in the chicken. Summerbell and Stirling (1981) exchanged left and right wing buds of the chicken between Hamburger and Hamilton stages 18 to 20, which is prior to axon entry to the limb (Bennett,

Davey and Uebel, 1980), thus reversing both dorso-ventral and anterior-posterior axes. In some cases extensor muscles were innervated by medial motoneurons, and flexors by lateral motoneurons, which is the converse of the normal pattern. One possible reason for this difference between the frog and chicken is that the rotation in chickens was made at the level of the shoulder, or elbow, which is distal to the branching of the main nerve trunks to the flexor and extensor muscle masses (Fig. 3. Stirling and Summerbell, 1977). In the frog, by contrast, the three segmental nerves form a single trunk prior to entering the hindlimb, and a rotation at that level would not be likely to restrict the availability of distal parts of the limb to growing axons.

Motor somatotopy:

The reasons as to why lumbar motoneurons can establish relatively specific connections with the muscles of a forelimb, and move the limb in co-ordination are speculative. Young (1972) found that serially homologous motoneurons in the mesothoracic ganglion of the cockroach selectively reinnervated the serial homologues of their own muscles in transplanted metathoracic limbs. Recently Wigston and Sanes (1982) have shown that two adult rat intercostal muscles are differentially innervated by autonomic axons at different segmental levels. Their suggestion that selectivity may depend on a recognition system that is widely distributed in the nervous system, based on relative position of nerves and muscles, is in accord with all of these findings.

In adults the relative positions of motoneuron somas within the spinal cord are characteristic of the particular limb muscle supplied (Romanes, 1964; Cruce, 1974 a; Hollyday, 1980 b; McHanwell and Biscoe 1981 b; Frank and Westerfield, 1982 a). Developmental changes in motor somatotopy have been observed in the projection to the *Xenopus* hindlimb (Lamb, 1976) and chicken forelimb (Pettigrew, Lindeman and Bennett, 1979), but not, to date, in the chick hindlimb (Landmesser, 1978 b; Lance-Jones and Landmesser, 1981 a; but see Lamb, 1981, b for comments). In *Xenopus* the changes in somatotopy are effected by motoneuron death (Lamb, 1977) and this may also occur in the early projection to the chick forelimb (Pettigrew *et al*, 1979). It should be noted, however, that at each rostrocaudal level of the spinal cord there are located motoneurons supplying several limb muscles, and when their dendrites are also taken into consideration the somatotopic organization is almost completely obscured. For example, in the frog spinal cord, each motoneuron's dendrites spread over almost the entire lateral funiculus at the level of the soma, and may extend for over 1000μm along the rostrocaudal axis (Székely, 1976). The developmental changes in somatotopy therefore imply, but do not necessarily prove the existence of 'erroneous' connections, as may be artificially induced by redirecting motor nerves in adults, since the central connections onto the motoneurons are not known. Conversely, normal motor somatotopy early in development may not necessarily show that the motoneurons have acquired central connections that are appropriate for the muscle in which each terminates, which is also true of motoneurons supplying rotated (Summerbell and Stirling, 1981) or supernumerary (Morris, 1978) limbs (Mark, 1980).

These studies, and the present observations therefore do not furnish evidence for peripherally induced changes of the central connections onto motoneurons during development, but these tests are presently limited by the lack of knowledge concerning the central neuron connections essential for co-ordinated limb movements.

Reflex behaviour of forelimbs innervated by lumbar segments:

As discussed by Hollyday and Mendell (1975), it is likely that cutaneous receptors mediate area-specific withdrawal reflexes in the *Xenopus* hindlimb, although it is possible that muscle receptors also contribute, or have their effect facilitated (Simpson, 1976) by the cutaneous afferent impulses. Wiping reflexes, which are mediated by cutaneous nerves (Baker, 1978) have been described in *Rana esculenta* (Franzisket, 1963), *R. pipiens* and *R. clamitans* (Miner, 1956) and *Discoglossus pictus* (Székely, Matesz, Baker and Antal, 1982). They have been used extensively in the amphibia to test whether the location of transplanted skin becomes appropriately, or otherwise, represented within the central nervous system (review by Baker, 1978). From such studies it has been shown that these reflexes, in contrast to the limb withdrawal reflexes, appear only after metamorphosis (Jacobson and Baker, 1969). They may be elicited in the spinal animal (Miner, 1956), and persist following deafferentation of the responding limb. This is also true of the homologous behaviour of scratching, in the cat (Sherrington, 1910; Berkinblit, Deliagina, Feldman, Gelfand and Orlovsky, 1978 a), and suggests that a central pattern generator located

within the spinal cord is responsible for the basic motor output (Berkinblit, Deliagina, Feldman, Gelfand and Orlovsky, 1978 b; Székely *et al*, 1982).

Although a number of experimenters have suggested that the periphery may exert some selective influence on the central connections of sensory neurons (see Introduction) the specificity of this effect has been a matter of controversy (see Mendell and Hollyday, 1976). Miner (1956) transplanted a hindlimb to the back of frog tadpoles, and tested reflex responses to cutaneous stimulation of the limbs after metamorphosis. She found that touching a transplanted limb produced a 'properly directed' reflex response in the normal ipsilateral limb, which in many cases was the forelimb, rather than the hindlimb. Similarly Székely and Szentágothai (1962) could evoke responses from a normal wing or leg of the chicken, by stimulation of a transplanted leg or wing, respectively, and this lack of specificity of the responses led them to reject the concept of peripheral specification for the cutaneous nerves supplying supernumerary limbs (see also Gaze, 1970). In repeating and extending these observations in *Xenopus*, Hollyday and Mendell (1975) described reflex responses that were specific to either the forelimb or hindlimb, and failed to elicit the typical pattern of hindlimb reflexes in the normal hindlimb, by stimulation of a transplanted forelimb innervated by lumbar segments. They suggested that the previous failures to distinguish between forelimb and hindlimb responses may have been due to stimulation of only the distal extremities, which produced flexion in either type of limb. It

was concluded that the homologous skin regions of the hindlimb or forelimb were unique in terms of the cutaneous reflex responses evoked from each.

The present observations of weak flexion responses of transplanted forelimbs suggests that sensory neurons are unable to establish strong reflex connections between the skin of a forelimb and the lumbar motoneurons. The finding that reflexes could not be evoked in the ipsilateral forelimb by stimulation of the transplanted forelimb furnished direct evidence for the suggestion of Hollyday and Mendell (1975) that a rostrocaudal limit might exist in the extent to which fibres from a transplanted limb could establish reflex connections with the homonymous motoneurons. Joseph and Whitlock (1968) traced degeneration by the Nauta method 4 segments rostral to the sectioned 9th spinal nerve in *Bufo marinus*, although some fibres ascended to the cerebellum.

In contrast to the sensory neurons innervating the transplanted forelimb, sensory neurons innervating trunk skin appeared to have established specific reflex connections with the lumbar motoneurons, despite the fact that these motoneurons were innervating a forelimb. This would be in accord with the retention of identity of the lumbar motoneurons as 'hindlimb' motoneurons, onto which specific reflex connections from trunk, but not forelimb skin, would form. Clearly it would be of interest to extend these observations electrophysiologically. This might be more difficult in the case of wiping reflexes, since Székely *et al* (1982) found that collaterals

from trunk skin nerves terminated entirely within thoracic segments, which implies that these reflexes are relayed, by one or more interneuron paths, to the motoneurons.

4.5 SUMMARY

1. The hindlimb bud of stage 49 tadpoles of *Xenopus laevis* was replaced with a stage 50 forelimb bud. The motor and reflex behaviour of the transplanted limbs was examined after metamorphosis.

2. All transplanted forelimbs moved in co-ordination with the hindlimb of the host during swimming, regardless of the limbs' orientation. Identification of lumbar motoneurons projecting to 3 specific regions of the forelimb provided some evidence of selective innervation. These observations did not furnish evidence of a peripheral influence on the acquisition of central connections by the lumbar motoneurons.

3. The reflex withdrawal of the normal forelimb was not accompanied by withdrawal of the transplanted forelimb. Swimming could be elicited by a strong stimulus delivered to any part of the skin. Mechanical stimulation of the skin of the transplanted forelimb elicited a small flexion response in the limb, unaccompanied by withdrawal reflexes in other limbs, as judged behaviourally.

4. Wiping reflexes could be elicited in *Xenopus* by noxious chemicals delivered to the skin. Chemical stimuli applied to the area of skin normally wiped by the hindlimb, elicited movements in transplanted forelimbs.

5. These observations suggest that reflex connections may be made between the skin of the trunk and lumbar motoneurons supplying a

transplanted forelimb, but not between the skin of a transplanted forelimb and the lumbar motoneurons, nor the brachial motoneurons when the forelimb was in the hindlimb position.

CHAPTER FIVE

EARLY IONIC EVENTS IN THE ACTION OF NERVE GROWTH FACTOR (NGF) ON A PHEOCHROMOCYTOMA CELL LINE (PC12)

5.1 INTRODUCTION*

In studying the effect of limb amputation on the lateral motor column in *Xenopus laevis*, Prestige (1967 a) defined three phases in the maturation of the motoneurons. Limb amputation during the earliest development phase (I) had no effect on the neurons, during phase II it caused cell degeneration within three days, and during phase III chromatolysis took place, followed by degeneration after several weeks or more. This led to the proposal that cells in phases II and III died because they did not receive a maintenance factor carried from the limb via the axons, which could be stored as the neuron matured.

During 1981 I obtained a fellowship which enabled me to study molecular methods in embryology. The project chosen is relevant to the preceding work in that the 'classical' embryological work described implies the existence of a retrogradely transported motoneuron maintenance factor. Nerve growth factor is the best known of these and knowledge of its biochemical modes of action is certainly pertinent to how survival factors for other developing systems might operate. This work is introduced below.

*Part of the work reported in this chapter was carried out in conjunction with Drs. J. Boonstra, W. Moolenaar and S. de Laat, Hubrecht Laboratory, International Embryological Institute, Uppsalalaan 8, 3584CT, Utrecht, the Netherlands. I express appreciation for the instruction and use of facilities there.

Nerve growth factor (NGF) is a polypeptide essential for the survival of DRG and sympathetic ganglion neurons in vitro (reviewed by Varon and Adler, 1980, 1981). NGF is retrogradely transported from the chick hindlimb to the lumbar DRG on the 10th day of incubation, during the natural period of death of DRG neurons (Brunso-Behtold and Hamburger, 1978), and injections of NGF into the yolk sac of embryos from 3½ days onwards greatly reduced the number of DRG neurons dying. In combination these findings suggested that NGF may serve as a maintenance factor for DRG neurons during normal development (Hamburger, Brunso-Bechtold and Yip, 1981).

NGF first acts by binding to specific receptors on the cell membrane, and in common with various other growth factors (Rozengurt and Mendoza, 1980), its rapid effects include changes in monovalent ion fluxes across the cell membrane (Skaper and Varon, 1979, 1981). In particular, intact chick DRG (8 days) lose their ability to regulate intracellular Na^+, K^+ levels when deprived of NGF for several hours, but recover within minutes of NGF presentation (Skaper and Varon, 1979).

PC 12 is a noradrenergic cell line established from a rat pheochromocytoma (Greene and Tischler, 1976; Dichter, Tischler and Greene, 1977). In medium containing horse serum the cells undergo division (Fig. 5.1 a). With NGF treatment division ceases, and the cells express many of the differentiated properties of adrenal chromaffin cells (Fig. 5.1 b), including electrical excitability (Dichter, Tischler and Greene, 1977), and storage and synthesis

of catecholamines (Greene and Tischler, 1976). These changes take place over a period of several days. When serum is withdrawn, 90% of PC 12 cells die within 4 - 6 days, however NGF will maintain the surviving cells for at least a month. The remaining cells die upon NGF withdrawal (Greene, 1978).

It has been shown that NGF stimulates the Na^+-K^+ pump mediated K^+ influx in PC 12 cells, this change being evident within 15 minutes after NGF presentation (Boonstra, van der Saag, Moolenaar and de Laat, 1981). In this study the ionic events underlying this response were further investigated, with emphasis on their relation to the effects of other growth factors.

5.2 METHODS

Cell culture: PC 12 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum and 5% heat-inactivated horse serum at 37°C and 5% CO_2.

For flux experiments cells were plated at a density of 30,000 cells/cm^2 in culture dishes treated with poly-l-lysine (3.5 cm diameter) and grown over a period of 2 - 3 days to a final density of about 40,000 cells/cm^2.

Influx measurements: The growth medium was replaced with DMEM without serum for at least 2 hours prior to measurement. For estimates of K^+ influx, $^{86}Rb^+$ was used as a tracer for K^+, due to its longer half-life.

(The initial rate of uptake of the isotope is linear over a 5 minute period, see Boonstra *et al.* 1981). $^{86}Rb^{+}$ was added to the medium at a final concentration of 2-5 nM (spec. act. 1 Ci/mole K^{+}) and the cells incubated for 5 minutes at 37°C. The labelled medium was then removed and the cells washed 5 times in ice-cold phosphate buffered saline (PBS). Washed cells were lysed in 10% TCA and the lysates transferred to a scintillation vial. Samples were counted in a Packard scintillation counter, with 4 minutes measurement per vial. Corrections for isotope trapped in the extracellular space were made by washing the cells 5 times immediately after addition of the $^{86}Rb^{+}$. When used, ouabain at 37°C was added immediately prior to addition of the tracer, at 5 mM final concentration.

Na^{+} influx was measured with $^{24}Na^{+}$ as a tracer as described above, however the $^{24}Na^{+}$ was added in the presence of ouabain (5 mM) to prevent $^{24}Na^{+}$ efflux, and the cells were dissolved in 1N NaOH and counting conducted in Lumagel scintillation fluid in a liquid scintillation counter. The initial rate of Na^{+} uptake was linear, in the presence or absence of 5 mM ouabain. Sodium counts were corrected for isotope decay during counting (2 minutes counting per vial), assuming simple logarithmic decay.

Cell counting: At the conclusion of each experiment several culture dishes were washed 5 times in ice-cold PBS, 1 ml of 10% trypsin was added for 5 minutes, and the cells were ejected several times through a large gauge syringe to break up clumps, and counted under the microscope.

NGF was added to the cells at a final concentration of 50mg/ml of medium in all experiments at zero time.

Chemicals: 2.5s NGF ultrapure (isolated from mouse submaxillary gland) Batch No 11-B-80
Cadempino - Ti Switzerland

ouabain octahydrate Batch No 0-3125
Sigma, St. Louis

amiloride (3,5-diamine-6-chlorpyraginoyl-guanidine hydrochloride) Batch No 684 L 044
Merck, Sharp and Dohme, Haarlem, N.L.

poly-l-lysine Sigma, St. Louis

$^{86}RbCl$ (specific activity 1 Ci/mole K^+) The Radiochemical Centre, Amersham, England.

$^{24}NaCl$

heat inactivated horse serum Flow Laboratories

fetal calf serum.

The diuretic amiloride inhibits Na^+/H^+ exchange across the cell membrane - see Moolenaar *et al.*, 1981.

5.3 RESULTS

Rate of $^{24}Na^+$ uptake by PC 12 cells in response to nerve growth factor (NGF) treatment:

Sodium uptake by PC 12 cells in response to NGF (50ng/ml final conc.) was measured using a 5 minute pulse of $^{24}Na^+$. It was previously determined that the rate of Na^+ uptake was linear over this period. Ouabain at a final concentration of 5mM was used to block active Na^+ efflux. The cells were deprived of serum for 2 hours prior to measurement.

An almost 2-fold stimulation of Na^+ influx was observed within 15 minutes of NGF presentation (Figure 5.2). This rate of influx was maintained over the 90 minute period studied, without evidence of a decline. There was a small, but insignificant increase in control (-NGF) Na^+ influx during the 90 minute period.

Serum stimulation of the Na^+-K^+ pump in PC 12: Activity of the Na^+-K^+ pump was estimated from ouabain inhibitable K^+ influx measurements, using a 5 minute pulse of $^{86}Rb^+$ as a tracer for K^+. It had earlier been determined that the initial rate of uptake of $^{86}Rb^+$ was linear over this period.

Following 2 hours of serum deprivation (Figure 5.3) the pump-mediated K^+ influx was estimated to be 3.60±0.35 nmoles K^+/min/10^6 cells, N = 9) in the presence of the diuretic amiloride (0.2 mM final conc.). Thirty minutes after addition of serum (15%),

FIGURE 5.1 PC12 CELLS IN CULTURE BEFORE, AND AFTER, 24 HOURS EXPOSURE TO NGF

Cells were grown in DMEM as described in Methods, in the presence of 15% serum (A), or NGF at a final concentration of 50ng/ml. Medium was removed after 24 hours, and photos were taken 24 hours after addition. Some cells in (B) have begun to extend neurites.

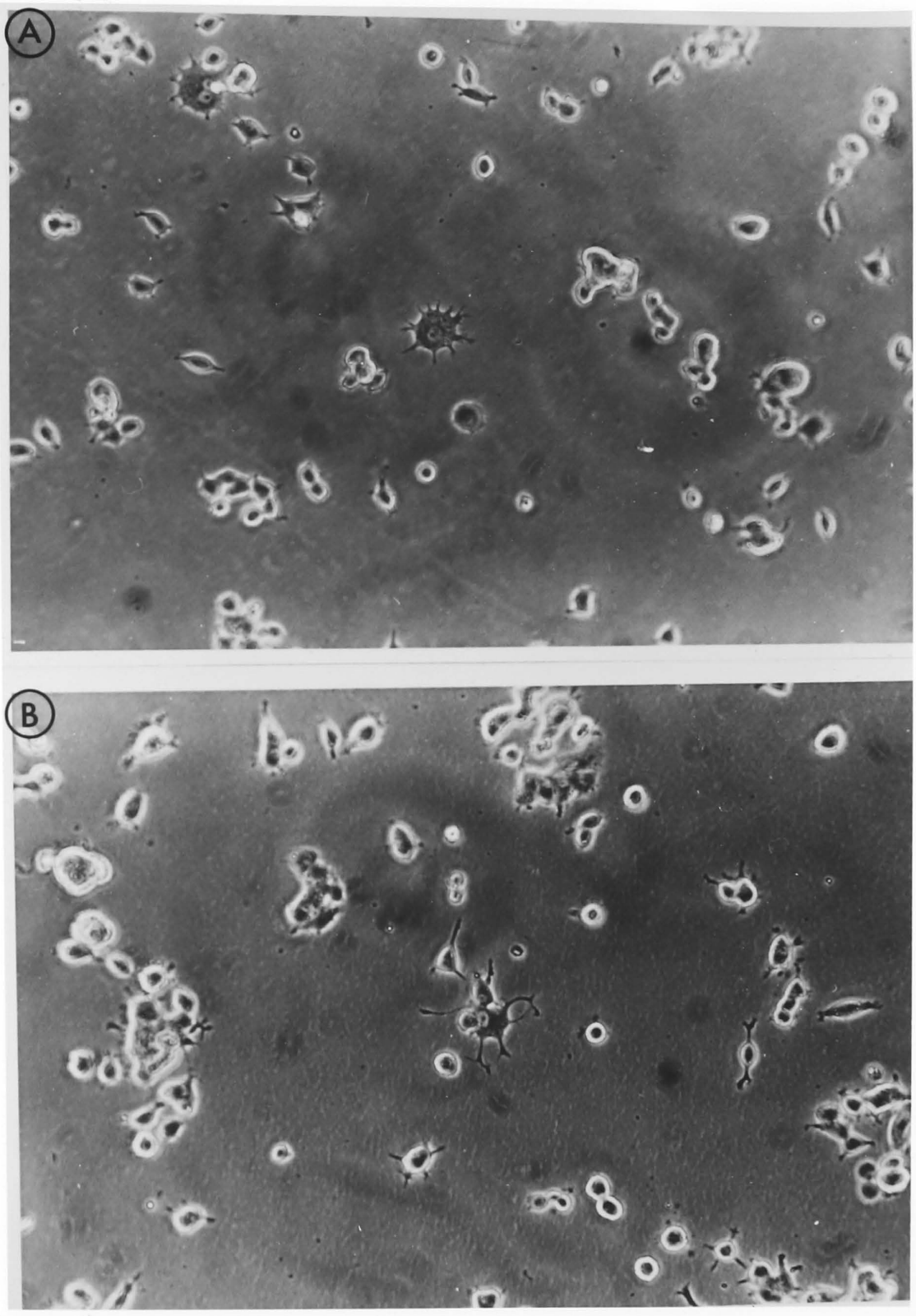

A
B

FIGURE 5.2 RATE OF $^{24}Na^{+}$ UPTAKE BY PC12 CELLS IN RESPONSE TO NERVE GROWTH FACTOR (NGF) TREATMENT

The rate was determined by a 5 minute pulse of $^{24}Na^{+}$ added to the extracellular medium. NGF concentration was 50ng/ml medium

Data presented as mean $\pm$ SEM (N = 6)

Control - lower trace; Experimental - upper trace.

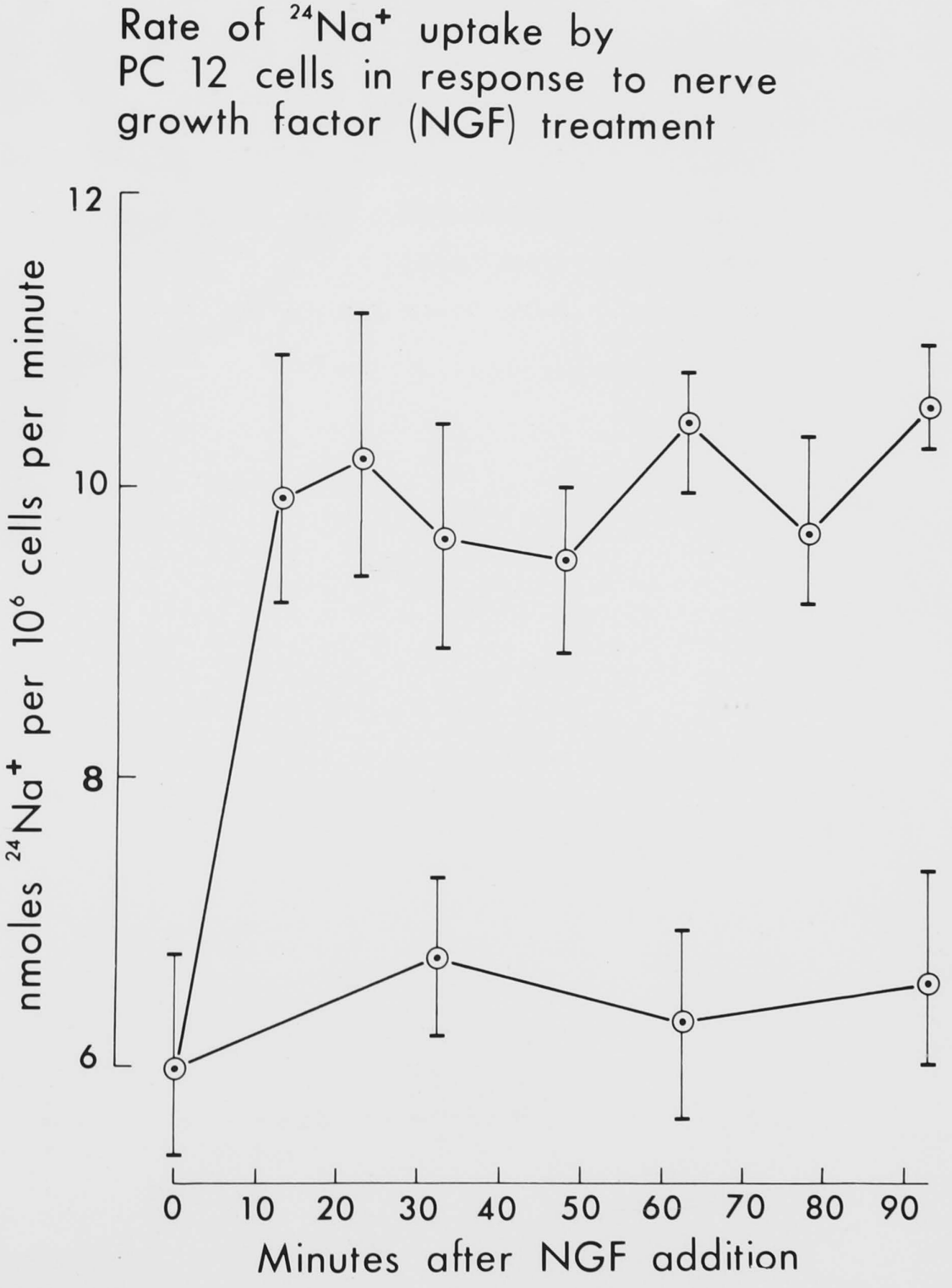
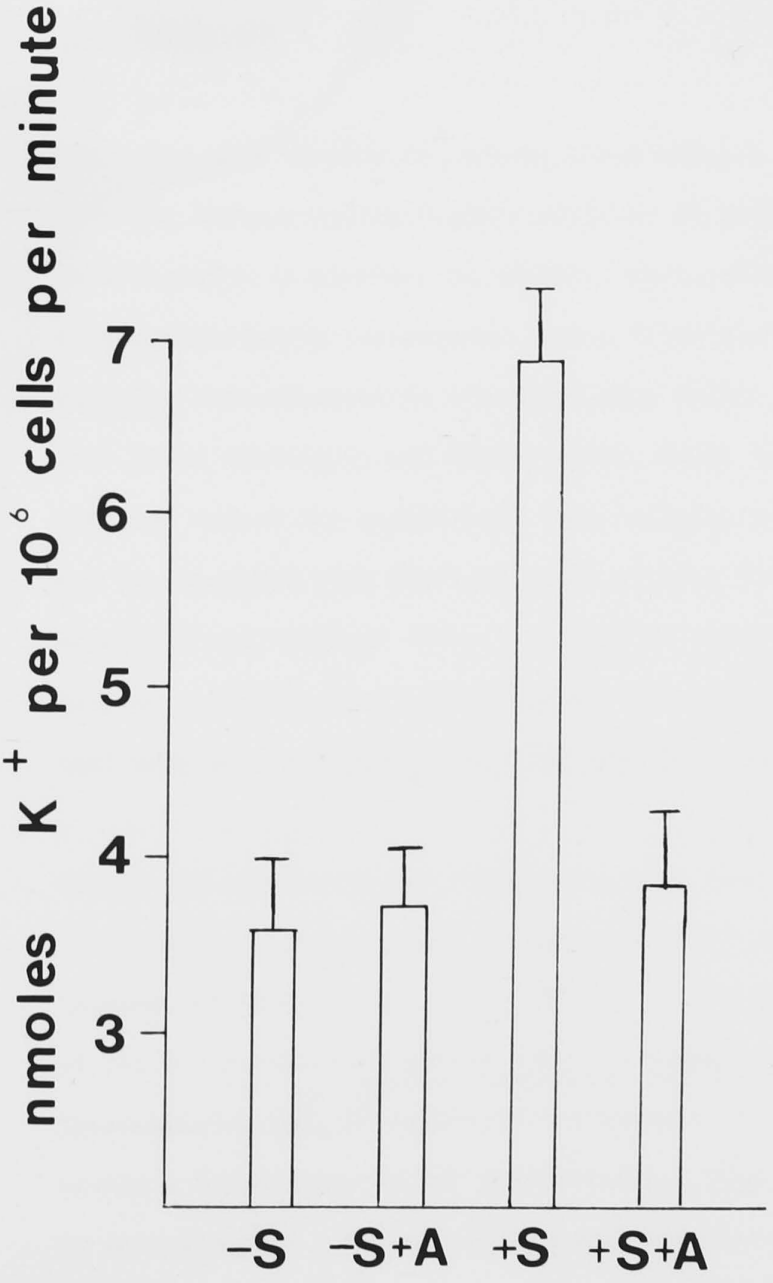

Rate of $^{24}Na^{+}$ uptake by PC 12 cells in response to nerve growth factor (NGF) treatment
nmoles $^{24}Na^{+}$ per 10^{6} cells per minute
12
10
8
6
0
10
20
30
40
50
60
70
80
90
Minutes after NGF addition

FIGURE 5.3 EFFECT OF SERUM (S) + AMILORIDE (A) ON $Na^+ - K^+$ PUMP-MEDIATED K^+ INFLUX

K^+ pump-mediated influx was measured at 37°C as described in Methods. Serum (S) at concentration 15% or amiloride at final concentration 0.3mM were added to the cells 10 minutes prior to addition of the Rb^+ tracer.

Data presented as mean ± SEM (N = 6).

Effect of serum (S) ± amiloride (A)
on $Na^+ - K^+$ pump - mediated K^+ influx



the pump rate was elevated almost 2-fold, to 6.90±0.40 nmoles K^+/ min/10^6 cells (N = 9). This increase was almost entirely abolished in the presence of 0.2 mM amiloride, to 3.81±0.41 nmoles K^+/min/10^6 cells, (N = 8).

5.4 DISCUSSION

The cells of a variety of culture lines enter a 'quiescent' phase when the culture medium becomes depleted of growth factors, and cell division is minimal, or absent. Upon addition of fresh serum, cell proliferation recommences, and a stimulation of the Na^+-K^+ pump has been observed to accompany this effect, in a variety of cell lines (Rozengurt and Heppel, 1975; Smith and Rozengurt, 1978). This has raised the possibility that changing monovalent ion fluxes may be concerned with the initiation of cell division (review by Rozengurt and Mendoza, 1980), although the intermediate events between the binding of growth factors to specific receptors on the cell membrane, and subsequent cell division, are largely unknown.

Changes in monovalent ion fluxes have also been shown to be involved in the early actions of NGF on DRG neurons of the chick embryo (Skaper and Varon, 1979, 1980, 1981). Intact and dissociated DRG of the 8 day-old chick embryo lose the ability to regulate intracellular Na^+, K^+ levels in the absence of NGF, but recover within a few minutes of NGF presentation. This probably takes place by activation of a Na^+,K^+ pump (Skaper and Varon, 1980). This response gradually becomes an indigenous property of the cells as they increase in developmental age (Skaper, Selak and Varon, 1982).

More recently it has been shown that Na^+-K^+ pump stimulation by serum growth factors is due to an early increase in local Na^+ influx, in quiescent fibroblasts (Smith and Rozengurt, 1978), in hepatocytes (Koch and Leffert, 1979) and mouse neuroblastoma cells (Moolenaar, Mummery, P. van der Saag and S. de Laat, 1981). Thus the effect of serum growth factors may be mimicked by agents that increase Na^+ influx, such as the ionophore monensin (Moolenar *et al* 1981), or melittin, (Rozengurt, Gelehrter, Legg and Pettican, 1981). Furthermore, the Na^+ influx, stimulation of the Na^+-K^+ pump, and division of neuroblastoma cells are all inhibited by the diuretic amiloride (Moolenaar *et al*, 1981). This drug is known to block electroneutral Na^+/H^+ exchange in other cell types (Aickin and Thomas, 1977), and raises the possibility that Na^+/H^+ exchange, and consequently changes in the intracellular pH, are involved in the stimulation of cell proliferation by growth factors.

In the present study it was shown that in addition to NGF, serum also stimulates the Na^+-K^+ pump in serum-deprived PC 12 cells, and that this effect is inhibited by amiloride. This serum response is not surprising in view of the fact that PC 12 cells differ from normal sympathetic neurons in that their differentiation to the neuronal phenotype can be reversed, and that in the absence of NGF the cells possess membrane receptors for epidermal growth factor (Huff, End and Guroff, 1981).

It remains to be determined whether either of the effects of NGF or serum are caused by an enhanced Na^+ influx, and whether PC 12 cells possess a Na^+-H^+ exchange mechanism. This possibility is certainly raised by the observed amiloride sensitivity of the serum-stimulation of the Na^+-K^+ pump. If that were so, then the early effects of NGF could be regarded not as a unique cellular response, but as part of a general set of trophic responses of cells to factors required for their survival and growth (Huff, *et al*, 1981). Cell survival and growth, and as a consequence, cell differentiation might then be inextricably linked.

5.5 SUMMARY

1. When deprived of serum, cells of the rat pheochromocytoma cell line PC 12 die, unless provided with the chemical nerve growth factor (NGF). The initial rate of uptake of $^{24}Na^{+}$ in response to NGF was shown to increase almost 2-fold over a period of 90 minutes after NGF presentation to serum-deprived PC 12 cells. This may be the basis of the known effect of NGF in stimulating the Na^{+}-K^{+} pump in this cell line.

2. It was shown that in addition to NGF, serum also stimulated the Na^{+}-K^{+} pump activity, and the stimulation could be reduced by the diuretic amiloride. It was concluded that some of the ionic events initiated by NGF in this cell line were common to other growth factors.

CHAPTER SIX

CONCLUDING REMARKS

In Chapter One the literature concerning the specification of spinal neuron connections was reviewed. Largely for reasons of technical convenience, most experiments concerning the subject have been conducted on the neuromuscular and somatosensory systems. There has also been an exchange of concepts with work in the retinotectal system, in the belief that general principles of neural development exist. Whereas studies of normal animals inform us of the succession of events during development, experimental manipulations, as in the present study, are necessary to determine why particular events occur. Reinnervation studies have to a large extent guided the approach to experimental studies of development, and deserve critical examination with respect to the results presented here.

1. The central connectivity of motoneurons during reinnervation and development

It is obvious that each limb muscle performs a particular mechanical task in co-ordination with its neighbours. Early studies in comparative anatomy recorded the tendency for particular muscles to be supplied from particular segmental roots of the spinal cord, and by sectioning the cord at various levels the approximate location of motoneurons supplying axons to each root could be determined (Sherrington, 1892). Studies using retrograde chromatolysis, and transport of tracers, have confirmed and extended knowledge of this somatotopic organization.

Observations of the return of co-ordination to a forelimb grafted alongside the normal member, following transection of the brachial nerves (Weiss, 1936), led to the theory that the motoneurons could

selectively acquire central connections appropriate for the activation of any muscle which they happened to reinnervate (Sperry, 1941). A similar recovery of co-ordination was not evident in cases of nerve damage and regeneration in clinical studies, however (Sperry, 1945 a). When pairs of nerves or muscles in the hindlimb of the rat were surgically transposed, the animals did not regain co-ordination (Sperry, 1941, 1942), especially in situations requiring rapid, or reflex responses. This implied that although the motoneurons supplying different muscles might be indistinguishable morphologically, there were differences in the central connections received by each, which did not change significantly upon reinnervation of other muscles with different mechanical actions.

The connections onto motoneurons responsible for locomotion, breathing, scratching etc. are still largely unknown, but it has clearly been established in a wide variety of vertebrates that supraspinal and afferent inputs to the spinal cord may modulate, but are not the primary generators of rhythmic motor output (for locomotion see review by Grillner, 1975). In view of this central autonomy, it can be seen in retrospect that testing for changes in monosynaptic connections from muscle spindles onto homonymous and heteronymous motoneurons following peripheral nerve crosses (Eccles, Eccles, Shealy and Willis, 1962; Mendell and Scott, 1975) does not bear directly on the problem as to whether changes in the basic pattern of motor co-ordination can be influenced from the periphery in an adaptive way. These studies do show a loss of afferent connections onto axotomised motoneurons, however it has also been shown that locomotor output remains co-ordinated in the peripheral nerves of the cat's limb after the nerves have been ligated for up to 200 days (Gordon, Hoffer, Jhamandas and

Stein, 1980). The central connections mediating locomotion may therefore be more resistant to peripheral changes than the primary afferent connections.

It was first believed that the failure to produce adaptive central changes in mammalian, as opposed to urodele spinal cord, was due to a loss of developmental plasticity in the former group. Attention was therefore focussed on the lower vertebrates, and the failure to detect signs of degeneration in the cord, combined with an absence of recovery when innervation of foreign muscles could be assured (Mark, 1965), raised the possibility that selective reinnervation, rather than central reorganization was the mechanism of recovery. A number of studies have added further support to this explanation, by showing that the segmental territories of the axolotl limb nerves are restored following their surgical derangement and subsequent regeneration (Cass, Sutton and Mark, 1973; Bennett and Raftos, 1977). Again, these studies are not direct tests of myotypic respecification, however, since it would appear that the reinnervation of different muscles is only a transient situation during recovery.

Although it is assumed that the limb motoneurons differ in their central connectivity, we have little indication as to how these connections must be arranged throughout the motor columns, for proper function. Are the interneuron connections to each motoneuron pool unique, or are they common to several pools? The same question can be posed in the periphery, in terms of motoneuron 'identities'. Must the motoneurons connect with particular muscles, or in simple order across the flexor or extensor muscle masses, in the embryo?

The selectivity of muscular action in voluntary movements, and the specificity of muscle spindle and tendon organ afferents onto motoneurons suggest that the motor pool supplying each major limb muscle is unique, but the more fundamental connections responsible for basic motor routines involving the entire limb might not require such specificity.

This problem carries over to developmental studies, where several workers have reported changes in the somatotopy of the early motor projections to the limb (Lamb, 1976; McGrath and Bennett, 1979; Pettigrew, Lindeman and Bennett, 1979). As pointed out in Chapter Four, in adults the extensive ramifications of motoneuron dendrites almost completely obscures the somatotopic organization. This is well illustrated by the variations in location of motoneurons supplying supernumerary limbs. Despite shifts along the rostrocaudal axis of up to one segment in the position of motoneurons supplying the gastrocnemius muscle, the extra limb is still moved in coordination (Rubin and Mendell, 1980). The reported early changes in somatotopy therefore imply, but do not prove the existence of 'erroneous' or 'mismatched' connections during development, since the central connectivity of the motoneurons is unknown. Conversely, although most, or in some cases all (Landmesser, 1978 b) motoneurons appear to project in the same pattern as in the adult, there is the possibility that some of these motoneurons may have inappropriate central connections (Mark, 1980). There would then be no need to postulate the existence of small-scale errors to account for motoneuron death where the somatotopy appears normal (see Lamb, 1981 b).

The present study did not provide evidence for a peripheral influence on the connections acquired by lumbar motoneurons during development. In fact the tendency of motoneurons to connect with appropriate limb regions brings development into the province of the reinnervation studies. More direct tests must await increases in our knowledge of the central connections underlying motor behaviour. A relevant problem in the amphibian is the loss of primary motoneurons, innervating the myotomes, following birth of the secondary motoneurons which innervate the limbs. What becomes of the interneurons supplying the primary motoneurons? Do they also regress, or do they reconnect with the newly generated limb motoneurons? This problem may be accessible to contemporary analytical techniques.

The failure to influence the central connectivity of the lumbar motoneurons innervating the transplanted forelimb was also shown in terms of the limb movements elicited by cutaneous stimuli delivered in the hindlimb wiping reflexogenous zone. The establishment of reflex connections between the skin of the trunk and the lumbar motoneurons was not matched by the formation of strong reflex connections between the forelimb skin and lumbar motoneurons, however. One explanation of this result would be that, unlike the motoneurons, the sensory neurons of the dorsal root ganglia form central connections under some selective influence from the periphery. This has been suggested by Hollyday and Mendell (1975) and recently by Frank and Westerfield (1982 b), who have shown that early removal of the 2nd dorsal root ganglion, which normally supplies all the sensory innervation of the bullfrog forelimb, is followed by the formation of specific reflex connections from the arm onto the brachial motoneurons, by sensory

neurons in dorsal root ganglion 3. Since DRG 3 normally does not send axons into the forelimb, selective death of prespecified neurons is not likely to account for this result. Although the reflex responses elicited from heterotopic limbs have offered some support for a peripheral influence on the formation of central connections of sensory neurons, the specificity of this influence has been called into question (see Discussion, Chapter 4, also Székely, 1974). An electrophysiological study of the preparation described in this thesis would be likely to resolve this problem.

II. Chemospecificity, recognition and competition

Sperry (1963) proposed his now well known theory of chemoaffinity mainly to account for the recovery of visual functions following section and regeneration of the optic nerve in lower vertebrates. The theory proposed that the growing retinal axons possessed chemical labels that were graded in a simple pattern in relation to the geometry of the eye, and that the tectal neurons similarly carried a complementary set of chemical markers, which governed the affinity for synapse formation between the 2 sets. This scheme of matching was hypothesized to occur in other regions of the nervous system, and the only allowance for inaccuracies of axon growth during development was a process of induction through the terminal contacts, as was believed to occur with the motoneurons (Sperry, 1941). Thus the scheme was based on an absolute match, or recognition process between the pre- and post-synaptic neurons.

The theory has subsequently undergone one major revision, prompted by the so-called size disparity experiments, where fibres from half a retina have been allowed to grow onto a whole tectum, or from a complete retina onto a half tectum. It has been found that the fibres spread, or compress in an orderly manner so that no cells are left without forming, or receiving connections. Thus matching in this system was not an absolute recognition process, in the sense that there was a secondary mechanism concerned with equalizing the number of pre- to postsynaptic sites.

The nature of this matching process, which must involve interactions between presynaptic neurons once they have formed some connections with the postsynaptic target, is unclear in molecular terms, and doubt has even been expressed as to the existence of any form of marking of tectal neurons prior to the ingrowth of the axons from the retina (For a recent review see Keating, 1981; also Straznicky and Gaze, 1982, in relation to this point).

As discussed in Chapter One, reinnervation studies have shown that regenerating motor axons may form at least some synapses with any denervated muscle fibres they encounter. The stability of the synapses formed depends on 2 forms of competitive interaction between motoneurons. In all vertebrate groups studied the overriding competitive interaction appears to be a form of equalization of the number of contacts made by each motoneuron, irrespective of its central connections, which ensures that all pre- and postsynaptic cells form or receive synapses. The second form of competition has only been clearly displayed in the urodele amphibia, and involves competition between connections formed

by a different, and the original motor nerve within a muscle (Yip and Dennis, 1976; Bennett, McGrath and Davey, 1979). If the foreign terminals are not challenged for a period of over 10 weeks, they are not displaced by the original nerve (Bennett, McGrath and Davey, 1979). Matching therefore appears to be not an absolute, but conditional process in this system also.

It has also been suggested that this second form of competition might be of importance in the development of specific neuromuscular connections, following an early period of somewhat random outgrowth of motor axons (McGrath and Bennett, 1979; Pettigrew, Lindeman and Bennett, 1979; Mark, 1980). The ability of motoneurons to connect with appropriate muscles following rotation of the limb (Chapter Four), or reversal of several spinal cord segments (Lance-Jones and Landmesser, 1980) would suggest that a form of recognition of muscles by motoneurons, and vice-versa, also occurs during development. In fact the natural variations in the segmental supply to the limb, and the location of motoneurons supplying different muscles at each rostrocaudal level of the spinal cord would seem to require such a process. Whether recognition occurs by motoneurons initially sending axons to the appropriate muscles or limb regions, or by a selective loss of motoneurons that have unselectively connected with inappropriate regions, is not yet clear. The latter possibility has been raised by reports of changes in motor somatotopy of the projection to the frog himdlimb (Lamb, 1976) and chick wing (Pettigrew, Lindeman and Bennett, 1979) during early development, when a period of death eliminates over half of the motoneurons sending axons into the limb. In one case a developmental change of somatotopy has been shown to be effected

by motoneuron death (Lamb, 1977) although it is not known whether the neurons concerned actually form peripheral synapses.

Nonetheless changes in somatotopy have not been detected in the early projections to the chick hindlimb (Landmesser, 1978 b), and even in cases where they have been detected, by far the majority of motoneurons appears to project to appropriate limb regions, as defined by the adult pattern. A focus of interest in development has therefore been the selectivity, or otherwise of axon growth and termination. As mentioned in Chapter Two, although the limb nerve branching pattern appears to be determined by the limb, this does not rule out some selectivity of axon growth along those pathways. Indeed, axon outgrowth might take an intermediate form to the 2 extremes outlined above. It is not difficult to conceive of a step-wise retraction and extension of processes, as successive motor axons arrive or are lost during development, more akin to the situation during reinnervation.

The apparent difference between the development of innervation of the chick leg and wing is of importance, since the hindlimb has been used in several studies concerning selectivity, or otherwise, of axon growth (Lance-Jones and Landmesser, 1980, 1981 a, b). It should be noted that there exists a difference in the time course of motoneuron death in the lumbar as opposed to the brachial cord of the chick. Neuron death begins on about the 6th day of development in both regions, but is much more protracted in the brachial cord (Hamburger, 1975; Oppenheim and Majors-Willard, 1978). This distinction is also evident in the spinal cord of *Rana pipiens* (Pollack, 1969 a).

If changes in somatotopy occurred with the onset of innervation of the hindlimb then they might be expected to be more rapidly lost than those of the forelimb.

It has also been suggested that motoneuron death may be a means of quantitatively matching the numbers of pre- to postsynaptic cells (Cowan, 1973). Some evidence against this has been provided by Lamb (1980), where motor axons from both sides of the spinal cord were led into a single limb, and in some cases twice as many motoneurons as normal survived. In this study also, the number of surviving motoneurons was greater than would be expected according to this hypothesis. The elimination of polyneuronal innervation in newborn mammals also does not appear to be associated with motoneuron death (Lance-Jones, 1982). For further discussion see Lewis (*Nature*, 284: 305-306).

The major difficulty in testing any theory of competition between motoneurons during development is the lack of an independent means of identifying different motoneurons. It is possible to obtain a crude equivalent to the cutting and redirection of motor nerves possible in adults, however, by the transplantation of peripheral tissues, as performed in the current study. Indeed the first evidence for a competitive withdrawal of connections was provided by Hughes (1964 a, b) using such an approach. He grafted a forelimb alongside the hindlimb of the small amphibian *Eleutherodactylus*, and in doing so, cut the nerve supply to the hindlimb. Both limbs were reinnervated, presumably by motor collaterals branching to both limbs (see Stephenson, 1979), and were motile. The forelimb, however, became immobile towards hatching, suggesting a selective withdrawal of collaterals.

It has been shown in the present study that under non-competitive conditions (i.e. no hindlimb present), lumbar motoneurons can sustain normal levels of maturity into adulthood, whilst innervating a forelimb. This result weighs against absolute recognition of limb muscles by motoneurons during development, unless the basis of recognition was some property common to both the forelimb and hindlimb muscles, such as relative position in the early muscle mass.

A possible complementary experiment, which could provide evidence concerning competition between motoneurons, would be to transplant one or two segments of the brachial cord in between the lumbar segments. Provided that the transplanted motoneurons innervated the limb, their fate could be determined after development. Should competition between motoneurons be a means of ensuring the maintenance of the *qualitatively* appropriate connections, then the transplanted brachial motoneurons would not survive in the face of competition with lumbar motoneurons for the hindlimb muscles.

III. Trophic Factors

The survival of lumbar motoneurons following replacement of the hindlimb with a transplanted forelimb, but not myotomes, raises questions as to the survival requirements of developing neurons. The fact that many motor and sensory neurons die following amputation of the limb bud has led to the belief that the neurons may be dependent on chemical 'factors' which can only be obtained from their targets. This has naturally attracted considerable attention as a possible means of bridging the enormous gulf between events at the

molecular level, and at the level of the entire developing system. The effect of amputation on neuron survival does not in itself prove that such trophic factors exist, since other explanations can be forwarded, for example, young neurons might simply be more susceptible to the damaging effects of axotomy. It does seem likely, however, that NGF, and possibly other factors are involved in the early survival of DRG neurons (Hamburger, Brunso-Bechtold and Yip, 1981). If DRG neurons were dependent on NGF derived from the periphery, then this might explain the sensitivity of DRG neurons to sectioning of their peripheral, but not central processes (review by Cragg, 1970). It would also suggest that uptake of factors required for survival would not necessarily be through synapses, which are lacking in the peripheral connections of sensory neurons. Similarly with developing motoneurons, Prestige (1967 a) found that a small regenerating limb bud supported almost as many motoneurons as the normal hindlimb. Thus although the supply of factors might become restricted to the synaptic end plates on the muscle fibres, the number of synapses per-se might not be the limiting criterion by which some neurons, and not others, survive.

This also raises the question as to whether factors might be specific to particular targets, or target regions. If that were so, the factors and their antibodies would be useful tools in identifying and eliminating specific categories of neuron. In Chapter Five it was shown, however, that some of the early ionic effects of NGF in a pheochromocytoma cell line are held in common with other growth factors. The survival requirements of young neurons might not be unique, but may form part of a general set of trophic responses of

cells to growth factors, which during development may reach, or exceed critical levels, when in competition with other cells. This implies that the factors themselves may be relatively unspecific, while competition between neurons for the factors produces the highly specific interconnections evident in the adult.

A further question is the possible relation of factors involved in early neuron survival, and those believed to be involved in sprouting. The initial outgrowth of motor axons appears to be the result of a central 'drive' from the cell body, whereas the directed growth and sprouting of axons close to the target suggests that the target releases diffusible chemicals which influence the axons, and perhaps also the somas by retrograde transport of substances along the axons. The formation of synapses marks the beginning of an interaction whereby a fine balance is achieved between the pre- and postsynaptic cells. It would seem likely that the interactions which take such an extreme form during development, persist with some restrictions into the adult life of the organism.

While attention currently focusses on regions of the nervous system where neuron death occurs to a dramatic extent, the functional unity of the nervous system encourages the belief that the principles emerging will be of general application, perhaps even extending to the higher centres involved in perception and memory.

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